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Molecular cloning and regulation of the polymeric immunoglobulin receptor

Piskurich, Janet Francine, Ph.D.
Case Western Reserve University, 1994

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MOLECULAR CLONING AND REGULATION OF THE
POLYMERIC IMMUNOGLOBULIN RECEPTOR

by

JANET FRANCINE PISKURICH

Submitted in partial fulfillment of the requirements
for the Degree of Doctor of Philosophy

Thesis Advisor: Charlotte S. Kaetzel, Ph.D.

Department of Pathology

CASE WESTERN RESERVE UNIVERSITY

January, 1994
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MOLECULAR CLONING AND REGULATION OF THE
POLYMERIC IMMUNOGLOBULIN RECEPTOR

Abstract

by

JANET FRANCINE PISKURICH

Secretory IgA and secretory IgM, which substitutes for IgA in some IgA-deficient individuals, are the major immunoprotective factors operating in external secretions. These polymeric immunoglobulins are secreted locally by plasma cells, then transported across the epithelium by the polymeric immunoglobulin receptor (pIgR). pIgR, expressed on the mucosal and exocrine glandular epithelium, is cleaved and released during transport, becoming the secretory component (SC) that is an integral part of secretory immunoglobulins. Although it is clear that expression of pIgR protein can be modulated, little is known of the mechanisms involved. The purpose of this thesis work was to study mechanisms by which pIgR expression and release are regulated.

The effects of epithelial cell polarity on pIgR protein expression and SC release were studied using HT-29 human colon carcinoma cells. Upon depolarization, an initial burst followed by a decline in SC release was observed. This decline in SC release was not accompanied by a decrease in cell-associated pIgR. Cell
polarity plays a role in controlling SC release, possibly by segregating basolateral membrane-bound pIgR and apically localized pIgR protease.

Mouse and human cDNAs containing complete coding sequences of pIgR were isolated. A comparison of predicted amino acid sequences of mouse and human pIgR demonstrated that regions of the molecule implicated in IgA binding and epithelial transcytosis have been preferentially conserved.

To study the mechanism by which IFN-γ induces pIgR expression, pIgR mRNA was quantitated using a sensitive ribonuclease protection assay. PIGR mRNA increased in response to IFN-γ, reaching a plateau at 24 h. PIGR protein increased proportionally, but the major increase was not seen until 48 h after stimulation. Cycloheximide treatment abolished IFN-γ induced increases in pIgR mRNA, suggesting that induction of pIgR expression by IFN-γ requires de novo protein synthesis.

To identify DNA sequences involved in control of pIgR expression, the 5'-flanking region of the human pIgR gene was isolated and the start site of transcription was identified. Analysis of the sequence of the promoter proximal region revealed potential cis-acting regulatory elements similar to those found in MHC class II antigen genes.
DEDICATION

This thesis is dedicated to my father, the backyard geneticist, and my mother and brother. Their untimely passings have impressed upon me the fragility of life and the necessity to learn as much as we can possibly learn about it.
I would like to thank my advisor, Dr. Charlotte Kaetzel. If I have acquired even a small amount of her enthusiasm, optimism, and management and teaching skills from my association with her, I have been given a precious gift. I thank Dr. Michael Lamm for his support and guidance. I would like to express my thanks to the other members of my committee, Dr. John Nilson and Dr. Mark Tykocinski, not only for their advice and direction but also for sparking my interest in Molecular Biology and Immunology.

I would like to thank Dr. Alan Tartakoff and Dr. James Anderson for taking a chance on me, a student coming back to school after so many years. To the other graduate students, for accepting me and for their help, especially to: C. Kote Rao for philosophical discussions; John Hambor for answering all the questions I was afraid to ask anyone else; Carole Tamer for discussions on the "cell biology" of pIgR and for showing me that my techniques worked even when my experiments weren't working; Ken Youngman for his help on this project and for being a friend/enemy when I needed one.

I am grateful to the people I work with: Thomas Blanchard, David Fletcher, Janet Robinson, Norma Sigmund; and especially, John France and May Hsieh. This project could never have been
completed without their help. I would also like to thank my Macintosh advisors: Dr. John Nedrud and Ken Youngman.

It is impossible to acknowledge all of the individuals who have helped me on this voyage once around the world of science and ready to begin again. There are many who are not mentioned here but who will be remembered in tales on future voyages. You are the stuff of which modern legends should be made.

Finally, I would like to acknowledge the love and devotion of my husband. Without him...
### TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abstract</td>
<td>ii</td>
</tr>
<tr>
<td>Dedication</td>
<td>iv</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>v</td>
</tr>
<tr>
<td>Table of Contents</td>
<td>vii</td>
</tr>
<tr>
<td>List of Figures</td>
<td>x</td>
</tr>
<tr>
<td>List of Tables</td>
<td>xii</td>
</tr>
<tr>
<td>Abbreviations</td>
<td>xiii</td>
</tr>
<tr>
<td>Chapter 1. Introduction</td>
<td>1</td>
</tr>
<tr>
<td>Mucosal immunity and the role of secretory immunoglobulins</td>
<td>2</td>
</tr>
<tr>
<td>The polymeric immunoglobulin receptor undergoes transcytosis in polarized epithelial cells</td>
<td>8</td>
</tr>
<tr>
<td>P1gR protein and gene structure</td>
<td>11</td>
</tr>
<tr>
<td>Regulation of P1gR expression</td>
<td>15</td>
</tr>
<tr>
<td>Regulation of MHC class II gene expression</td>
<td>19</td>
</tr>
<tr>
<td>Summary of results</td>
<td>23</td>
</tr>
<tr>
<td>Chapter 2. Cell polarity regulates the release of secretory component, the epithelial receptor for polymeric immunoglobulins, from the surface of HT-29 colon carcinoma cells</td>
<td>25</td>
</tr>
<tr>
<td>Abstract</td>
<td>26</td>
</tr>
<tr>
<td>Introduction</td>
<td>27</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>30</td>
</tr>
<tr>
<td>Cell culture</td>
<td>30</td>
</tr>
<tr>
<td>Enzyme-linked immunosorbent assay for SC</td>
<td>31</td>
</tr>
<tr>
<td>Treatment of HT-29.74 cells with low calcium media</td>
<td>32</td>
</tr>
<tr>
<td>Determination of cytosolic-free calcium</td>
<td>33</td>
</tr>
<tr>
<td>Results</td>
<td>34</td>
</tr>
<tr>
<td>Culture of HT-29.74 cells in low calcium medium disrupts cell polarity</td>
<td>34</td>
</tr>
</tbody>
</table>
Disruption of cell polarity is accompanied by changes in release of SC from the cell surface .......... 38
Effect of Ca++ analogs on release of SC ..................... 45
Discussion .................................................................. 47

Chapter 3. Molecular cloning of mouse and human polymeric immunoglobulin receptor cDNA .......... 52
Abstract ...................................................................... 53
Introduction .................................................................. 54
Materials and Methods ............................................. 56
  Cloning and sequencing of mouse and human pIgR cDNAs ......................................................... 56
  Isolation of murine intestinal epithelial cells ............................................................................. 61
  Northern blot analysis ............................................... 62
Results ......................................................................... 63
  Nucleotide sequences of mouse and human pIgR cDNAs .......................................................... 63
  Functional regions of the pIgR protein are highly conserved across species .............................. 67
  Mouse pIgR mRNA is expressed in liver and intestinal epithelial cells but not in spleen ............ 75
Discussion .................................................................... 76

Chapter 4. Interferon-γ induces polymeric immunoglobulin receptor mRNA in human intestinal epithelial cells by a protein synthesis dependent mechanism .......... 84
Abstract ...................................................................... 85
Introduction .................................................................. 85
Materials and Methods ............................................. 87
  Cell culture .................................................................. 87
  Measurement of cell-associated pIgR and released SC ............................................................... 88
  Measurement of pIgR mRNA ............................................. 88
Treatment of HT-29.74 cells with cycloheximide to block protein synthesis ............................. 91
Statistical analysis ....................................................... 91
Chapter 5. Molecular cloning of the promoter proximal region of the human polymeric immunoglobulin receptor gene

Abstract

Introduction

Materials and Methods

Isolation of human pIgR genomic clones

Southern blot analysis and DNA sequencing

Isolation of RNA from human intestinal epithelial cells

Primer extension analysis

Nuclease protection assay

Results

Identification of the start site of transcription of the human pIgR gene

The 5'-flanking region of the human pIgR gene

Discussion

Chapter 6. Discussion

References
<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1. The molecular structure of dimeric IgA and secretory IgA</td>
<td>5</td>
</tr>
<tr>
<td>1.2. Transcytosis of dimeric IgA by the polymeric immunoglobulin receptor transport system</td>
<td>10</td>
</tr>
<tr>
<td>1.3. The polymeric immunoglobulin receptor illustrated at the cell surface</td>
<td>13</td>
</tr>
<tr>
<td>1.4. General features of the promoter proximal region of the MHC class II genes</td>
<td>22</td>
</tr>
<tr>
<td>2.1. Transfer of HT-29.74 cells into low calcium medium results in changes in cell shape consistent with loss of cell polarity</td>
<td>37</td>
</tr>
<tr>
<td>2.2. Depolarization of HT-29.74 cells in low calcium medium is accompanied by a burst in release of SC</td>
<td>40</td>
</tr>
<tr>
<td>2.3. Effect of extracellular calcium concentration on SC release</td>
<td>44</td>
</tr>
<tr>
<td>2.4. Addition of Sr²⁺ to low calcium medium supports SC release</td>
<td>49</td>
</tr>
<tr>
<td>3.1. Nucleotide sequence of a mouse polymeric immunoglobulin receptor cDNA</td>
<td>65</td>
</tr>
<tr>
<td>3.2. Nucleotide sequence of a human polymeric immunoglobulin receptor cDNA</td>
<td>69</td>
</tr>
<tr>
<td>3.3. Interspecies alignment of polymeric immunoglobulin receptor amino acid sequences</td>
<td>73</td>
</tr>
<tr>
<td>3.4. Polymeric immunoglobulin receptor mRNA is expressed in mouse liver and intestinal epithelial cells</td>
<td>78</td>
</tr>
<tr>
<td>4.1. Dose-dependent induction of pIgR expression by IFN-γ</td>
<td>94</td>
</tr>
<tr>
<td>4.2. Ribonuclease protection assay for detection of pIgR mRNA</td>
<td>97</td>
</tr>
<tr>
<td>4.3. Quantification of pIgR mRNA by ribonuclease protection assay</td>
<td>99</td>
</tr>
<tr>
<td>4.4. Time course of induction of pIgR mRNA and protein by IFN-γ</td>
<td>102</td>
</tr>
<tr>
<td>4.5. Induction of pIgR mRNA is blocked by cycloheximide</td>
<td>105</td>
</tr>
</tbody>
</table>
Figure

5.1. Primer extension and nuclease protection analysis to identify the start site of transcription of the human pIgR gene.................................................. 120

5.2. The 5'-flanking region of the human pIgR gene........ 123

5.3. Comparison of the S, X and Y box sequences of the promoter proximal regions of the human pIgR and MHC class II genes.................................................. 128
<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1.</td>
<td>Effect of extracellular calcium concentration on expression and release of SC</td>
<td>42</td>
</tr>
<tr>
<td>2.2.</td>
<td>Effect of Ca(^{++}) analogs on release of SC</td>
<td>46</td>
</tr>
<tr>
<td>3.1.</td>
<td>Amino acid homology of pIgR is greatest in regions of the molecule involved in pIg binding and transcytosis</td>
<td>74</td>
</tr>
</tbody>
</table>
ABBREVIATIONS

bp base-pairs
CURL compartment for uncoupling of receptor and ligand
dIgA dimeric IgA
DTT dithiothrietol
fSC free secretory component
kb kilobases
LCa low calcium
MALT mucosa-associated lymphoid tissue
MDCK Madin-Darby canine kidney
min. minutes
mSC membrane secretory component
NCa normal calcium
nt nucleotides
PCR polymerase chain reaction
pIg polymeric immunoglobulin
pIgR polymeric immunoglobulin receptor
PIPES 1,4-piperazine diethanesulfonic acid
SC secretory component
sIg secretory Immunoglobulin
TGF transforming growth factor
CHAPTER 1
INTRODUCTION

Mucosal immunity and the role of secretory immunoglobulins

The mucosal immune system is the body's first line of defense against a hostile external environment. With the human gastrointestinal tract alone comprising some 200 to 300 square meters, the mucosal surfaces of the body are large and in intimate contact with an almost limitless variety of microorganisms, toxins, and dietary antigens. These surfaces are bathed by a host of humoral and cellular factors, allowing uptake of some substances while selectively inhibiting the penetration of others. Antibodies, the major class of protective factors operating in mucosal secretions, are supplied by a mucosal immune system whose function is distinct from the systemic immune system.

The independence of the mucosal immune system was proposed in 1919 by Besredka, who protected rabbits from a fatal bacterial dysentery by oral immunization and showed that protection did not correlate with serum antibody titers against the bacterium (1). Interest in mucosal immunity surfaced again in the 1960s when IgA was found to predominate in secretions, rather than IgG which is the predominant immunoglobulin class in serum (2). Several groups of investigators showed that plasma cells present at mucosal sites and in exocrine glands produce
mainly IgA (3, 4). Unlike the monomeric form which comprises 80-95% of the IgA in human serum, IgA that is secreted locally by mucosal plasma cells is predominantly dimeric (5), consisting of two IgA monomers covalently linked by a J (or joining) chain glycoprotein (6) (Fig. 1.1). In some IgA-deficient individuals, IgM-producing plasma cells predominate in the mucosa and pentameric IgM, also linked by J chain, becomes a major component in secretions and a major factor in mucosal immunity (7). This inherent redundancy points out the essential role played by polymeric immunoglobulins in secretions.

Secretory immunoglobulin is actually the product of two distinct cell types. A glycoprotein, known as secretory component (SC) was discovered in both secretory IgA (sIgA) and IgM (8-10). Secretory component became the clue that solved the mystery of the transport of these polymeric immunoglobulins across epithelia and into external secretions. In 1974, Brandtzaeg observed that a molecule sharing antigenic determinants with SC was present on the basolateral surface of secretory epithelial cells and proposed that SC was a receptor for polymeric immunoglobulin (pIg) (11). Mucosal plasma cells synthesize, assemble and secrete immunoglobulin polymers. Tight junctions between cells of the epithelia prevent diffusion of immunoglobulin into the mucosal lumen. To enter secretions, pIg must be transported across epithelia by receptors expressed on mucosal epithelial cells. SC is the soluble cleavage product of the membrane bound polymeric
Figure 1.1. The molecular structure of dimeric IgA and secretory IgA. Two IgA molecules linked by J chain form an IgA dimer. In secretory IgA, secretory component may form disulfide bonds with the Fc portion of one of the two IgA molecules.
A  Dimeric IgA:

B  Secretory IgA:
immunoglobulin receptor (pIgR), also called membrane secretory component (mSC) (12). It has been proposed that SC protects polymeric immunoglobulin from proteases and denaturants in the mucosal environment (13-15). SC is found in association with secretory but not serum IgA and IgM, which again emphasizes that the mucosal immune system is distinct from the systemic immune system.

The biological functions of secretory immunoglobulin have been extensively reviewed (16-18). SIgA acts as an immune barrier, preventing bacterial attachment (19, 20) and uptake of certain macromolecules including known carcinogens (21). Additional functions for mucosal IgA have been recently proposed. Neutralization of viruses by viral specific IgA passing through infected epithelial cells has recently been demonstrated (16). Also, an excretory function for sIgA has been proposed where pIgR functions to bind IgA immune complexes in the mucosal lamina propria and transport them into secretions, clearing them from the body (22). The biological role of secretory IgM has not been thoroughly investigated and the manner in which it substitutes for sIgA in some IgA-deficient individuals is not clearly understood (7, 23).

Regulation of mucosal immunity occurs in mucosa-associated lymphoid tissue (MALT) (17, 23). "Membrane" (M) cells are specialized epithelial cells which sample and import materials from the lumen into MALT for antigenic stimulation of B cells and
antigen presentation to T lymphocytes (17, 24). The magnitude of
the mucosal immune response may be related to variations in
MHC class II antigen expression (25). MHC class II determinants
are expressed not only on dendritic antigen presenting cells but
also on intestinal epithelial cells which may play a role in antigen
presentation leading to a systemic tolerance of dietary antigens
(26-28).

Antigen stimulated B cells, precommitted to IgA synthesis by
the local action of activated T cells, leave MALT and migrate to the
draining lymph nodes. After exiting the lymph, migrating B cells
enter the peripheral blood and are seeded to mucosal sites,
maturing into IgA-producing plasma cells only when acted upon
by locally released cytokines (IL-4, IL-5, IL-6, and transforming
growth factor-β (TGF-β)) (17). Lymphocyte migration is governed
by both cell-cell and cell-substrate interactions. Specialized
endothelial cells lining postcapillary venules express molecules
called "vascular addressins" which are recognized by cell adhesion
molecules referred to as "homing receptors" on lymphocytes (29).
Although the mechanisms involved in the generation of
lymphocytes with specific homing properties are unknown, it is
possible that environmental factors at the time of lymphocyte
activation may regulate homing behavior by regulating homing
factor expression (30). This migration process allows
dissemination of memory and effector cells to distant mucosal
sites providing body-wide surveillance.
The polymeric immunoglobulin receptor undergoes transcytosis in polarized cells.

The polymeric immunoglobulin transport system is one of the best-characterized examples of transcytosis (Fig. 1.2). PIgR is synthesized and core glycosylated in the rough endoplasmic reticulum of polarized epithelial cells, terminally glycosylated in the Golgi complex, and then inserted into the basolateral surface of these cells (34, 32). The immunoglobulin-receptor complex is endocytosed via coated pits and travels to a prelysosomal acidic endosomal compartment designated the "compartment for uncoupling of receptor and ligand" (CURL) (33). Although the receptor-ligand complex travels through CURL, polymeric immunoglobulin neither dissociates from its receptor nor does the receptor recycle. Instead, the complex is transcytosed across the epithelial cell. Release of the complex from the cell is preceded by a proteolytic cleavage of the receptor between its transmembrane and extracellular regions. This cleavage event occurs in close proximity to the apical cell surface (34, 35) and results in the release of secretory immunoglobulin, a complex of polymeric immunoglobulin and the extracellular portion of the receptor, from the apical surface of the epithelial cell into the mucosal lumen (36). The PIgR anchor is then internalized and degraded in lysosomes (35). In the absence of ligand, the receptor alone can be endocytosed, transcytosed and released from the cell as free secretory component (fSC) (36, 37). Although many secretions
Figure 1.2. Transcytosis of IgA by the polymeric immunoglobulin receptor (pIgR) transport system. After synthesis, pIgR localizes to the basolateral cell surface where dimeric IgA (dIgA) is available for association with the first domain of the extracellular region of pIgR. The immunoglobulin-receptor complex is internalized and travels by endocytosis to the apical cell surface. During transport, dIgA may form disulfide bonds with the fifth extracellular domain of pIgR. A cleavage event at the apical plasma membrane liberates the complex, allowing release of secretory IgA from the apical cell surface.
contain a large excess of fSC, the function of this fSC is unknown.

**PIgR protein and gene structure**

The primary structure of pIgR protein was first determined from the amino acid sequence of human fSC (38) and the deduced amino acid sequence of a full-length rabbit pIgR cDNA (39). Isolations of rat, mouse, and human pIgR cDNAs have now also been reported (40-43); see also Chapter 3). Like many surface glycoproteins, pIgR is a member of the immunoglobulin superfamily (44). Members of the Ig superfamily are constructed of domains, each about 100 amino acids long and folded into two β-sheets stabilized by a conserved disulfide bond. PIgR is composed of five extracellular segments which are related to Ig domains (Fig. 1.3). Four of these five Ig-like segments resemble Ig variable domains, but the fifth is more like an Ig constant domain. Ig-related molecules, like pIg and pIgR, have been shown to bind each other through homophilic interactions. It has been suggested that binding of pIg by pIgR may occur in stages, with the first stage involving noncovalent interaction of pIg with a conserved 23 amino acid region on the first Ig-like domain of pIgR (45, 46). Less avid interactions may then occur between pIg and other pIgR domains, bringing the two molecules in close alignment and facilitating the formation of a disulfide bond between pIg and the fifth domain of the receptor. Besides participation in the alignment of pIgR with polymeric
Figure 1.3. The polymeric immunoglobulin receptor illustrated at the cell surface. The putative site of the initial noncovalent interaction of polymeric immunoglobulin with the first extracellular domain of pIgR is shown. Immunoglobulin-like domains are shown by circles. The long cytoplasmic region of the receptor has been shown to contain signals that are important in receptor trafficking.
Polymeric immunoglobulin receptor:

Putative poly-Ig binding site

Ig-homology domains

Signals for intracellular trafficking

Extracellular | Transmembrane | Cytoplasmic
immunoglobulin, no other function for the second, third and fourth Ig-like domains of pIgR has been proposed. It is interesting that a functional form of rabbit pIgR has been identified, arising by alternative splicing of pIgR mRNA, that retains the pIgR-binding first, fourth and fifth Ig-like domains but lacks the second and third domains (47).

An analysis of all of the available pIgR cDNAs (39-43; see also Chapter 3) reveals several interesting features of the pIgR protein and the mRNA that encodes pIgR. In addition to the Ig-like domains, the complete pIgR protein sequence includes an 18 residue N-terminal signal peptide, a 21-23 amino acid transmembrane region, and a long (103 amino acids) cytoplasmic tail (see Fig. 3.3). The 5'-untranslated region (UTR) of the pIgR mRNA is long (>70 nucleotides) and the 3'-UTR is unusually long (>444 nucleotides). Involvement of these untranslated sequences in the regulation of receptor synthesis has not been investigated.

Several regions within the long cytoplasmic tail of pIgR have been implicated in the sequential localization of the receptor to the basolateral and apical cell surfaces (see Fig. 3.3). A 14-amino acid segment, adjacent to the transmembrane domain, appears to be required for basolateral localization (48, 49). In addition, the C-terminal 30 amino acids of pIgR have been shown to control the rate of internalization of the receptor (50). There is evidence that phosphorylation of serine residues within each of these regions plays a role in receptor trafficking (51, 52). Phosphorylation of
the serine residue within the membrane adjacent segment appears to be essential for entry of unoccupied pIgR into the transcytotic pathway. Transcytosis of pIgR that had a single amino acid substitution at this site was inhibited only in the absence of dIgA (52). Binding of the natural dIgA ligand rescued the mutant receptor. It is possible that dIgA binding may produce a conformational change in pIgR which enhances transcytosis.

Human pIgR is encoded by a single gene located on the long arm of chromosome 1 (region q31-q41) (42, 53). The gene structure of proteins belonging to the Ig superfamily (44) usually conforms to the "one-domain/one-exon" rule. An analysis of the human (54) and rabbit (47) pIgR genes revealed that three of the five Ig-like domains of pIgR lie on separate exons. The second and third Ig-like domains are encoded on a single exon which is involved in alternative splicing of the rabbit gene.

**Regulation of pIgR expression**

Both polyclonal and monoclonal antibodies generated against free secretory component have been used to study the tissue localization of pIgR protein expression. PIgR is expressed on the sinusoidal surface of hepatocytes in many rodent species (55). In rodents, polymeric IgA circulates and is transported from blood into bile by pIgR (56). Circulating IgA in humans is primarily monomeric and the expression of pIgR in the human liver is
limited to the hepatic biliary epithelium (57). The receptor is also expressed in a wide variety of epithelial tissues. In the small intestine, pIgR expression is greatest in columnar cells in the lower portion of the crypts, while MHC class II antigen expression is greater in the upper portions of the crypts where the more mature enterocytes are found (23). In the colon, the receptor is expressed in both crypt and surface epithelium. Metaplastic crypt columnar cells show decreased pIgR expression (58). In gastric mucosa, the antral and isthmsus zones have the highest levels of receptor expression (23). pIgR expression is increased in chronic gastritis and in other inflammatory lesions including celiac disease, Crohn's disease, ulcerative colitis and Sjogren's syndrome (23, 59). Increases in expression of pIgR in diseased epithelia are usually accompanied by aberrant increases in MHC class II expression (60). These findings may reflect enhancement of both pIgR and MHC class II expression in response to lymphokines secreted locally by activated T cells (see below).

The immunohistochemical findings on the tissue distribution of pIgR expression have been extended by Northern analysis of pIgR mRNA. A single pIgR mRNA of approximately 3.8 kilobases (kb) has been demonstrated in human duodenum, jejunum and colon (41). Low levels of pIgR mRNA were detected in human liver, probably reflecting the expression of pIgR by the biliary ductal epithelium. A single rat pIgR mRNA of approximately 3.5 kb (40) and two rabbit pIgR transcripts (~3.8 kb and ~3.1 kb) (47).
arising by alternative splicing, were detected in liver in these species.

The HT-29 human colon carcinoma cell line has been used as a model system to study the regulation of expression of pIgR in human intestinal epithelium. HT-29 cells have been shown to undergo reversible structural and functional enterocytic differentiation when grown in glucose-free medium (61). HT-29 cells express the pIgR on their surfaces and transport polymeric immunoglobulin (62). However, uncloned HT-29 cells are heterogeneous in their expression of pIgR (12). HT-29.74 is an HT-29 subline cloned by our laboratory and chosen for increased expression of pIgR and release of fSC into culture medium (63). HT-29.74 cells express dramatically increased levels of the protein as measured by ELISA when induced to differentiate by glucose deprivation (64). The molecular weight of the fSC released by these cells is consistent with the characteristic proteolytic processing of pIgR.

The production of cytokines at mucosal sites may serve to enhance the efferent limb of the secretory immune system by increasing pIgR expression and secretory immunoglobulin production. PIgR expression in human colonic epithelial cells has been shown to be regulated by IFN-γ, tumor necrosis factor-α (TNF-α) and IL-4 (65-68). IFN-γ, produced by activated T cells and NK cells, induces expression of a large number of proteins, including many immunoglobulin gene superfamily proteins such
as: MHC class I and class II antigens, MHC class II invariant chain, \( \beta_2 \)-microglobulin, Fc\( \gamma \)R and pIgR (69). IFN-\( \gamma \) has been reported to enhance pIgR protein expression in both undifferentiated and differentiated HT-29 cells (43, 65; see also Fig. 4.1). Increases in pIgR expression have been shown to be accompanied by increases in the expression of MHC class II antigens, \( \beta_2 \)-microglobulin and Fc\( \gamma \)R (65). Enhancement of pIgR expression by IFN-\( \gamma \) involves an increase in the steady state concentration of pIgR mRNA (43, 70; see also Fig. 4.4). Cycloheximide abolishes the IFN-\( \gamma \) induced accumulation of pIgR mRNA (43, 70; see also Fig. 4.5), suggesting that pIgR induction requires de novo protein synthesis.

Two other cytokines, TNF-\( \alpha \) and IL-4, have been shown to enhance pIgR expression in HT-29 cells (66-68). The effects of IFN-\( \gamma \) and TNF-\( \alpha \) on pIgR expression in HT-29 cells were additive (67). MHC class II antigens were also additively induced in these cells by IFN-\( \gamma \) and TNF-\( \alpha \). TNF-\( \alpha \) is produced mainly by activated macrophages (71) which are present in large numbers within mucous membranes (72). TNF-\( \alpha \) has been shown to activate the transcription factor NF-\( \kappa \)B, and also to bring about the de novo synthesis of several other transcription factors, including AP-1, IRF-1 and IRF-2 (73).

Synergistic stimulations of pIgR expression by IFN-\( \gamma \) and IL-4, and by all three cytokines (IFN-\( \gamma \), TNF-\( \alpha \) and IL-4) were observed in HT-29 cells (68, 74). Stimulated human intestinal mononuclear cells produce culture supernatants containing these
cytokines. These supernatants were shown to be potent inducers of pIgR expression for human colonic epithelial cells (74).

**Regulation of MHC class II gene expression**

The observation that enhancement of pIgR expression by IFN-γ is accompanied by parallel increases in MHC class II antigen expression suggests that the regulatory mechanisms of these genes may have features in common. The MHC class II or Immune-associated (Ia) antigens are cell surface glycoproteins encoded by a family of genes located in the major histocompatibility complex on chromosome 17 in mice and on the short arm of chromosome 6 in humans. Different cell types express these antigens either constitutively, inducibly, or not at all (75). There have been reports of IFN-γ induced increases in steady-state mRNA levels for MHC class II antigens in many nonlymphoid cell types including a human glioblastoma multiforme cell line (76), and primary cultures of human astrocytes (77), endothelial cells and dermal fibroblasts (78). Nuclear runoff assays have been used to demonstrate transcriptional rate enhancement of MHC class II genes by IFN-γ (79). However, this enhancement was insufficient to explain the total increase observed in MHC class II mRNA. It has therefore been suggested that IFN-γ increases mRNA levels for MHC class II gene products by more than one mechanism, possibly including stabilization of MHC class II transcripts (75, 80).
Transcriptional regulation of MHC class II genes has been studied extensively (81-83). Several common cis-acting elements which were originally identified by sequence comparisons of the MHC class II genes have been characterized (Fig. 1.4). These promoter/enhancer elements have been studied using a variety of techniques including transgenic mouse models, transient transfection of reporter constructs, electrophoretic mobility shift assays, and in vitro and in vivo DNA footprinting analyses.

The MHC class II gene promoter proximal region (-180 to +10 base-pairs (bp) relative to the transcriptional start site) has been shown to contain several sequence motifs which regulate gene expression. Promoter function requires the X and Y boxes (Fig. 1.4) to be present and their spacing (18-20 bp, two helical turns) must be maintained. An X2 region, resembling a cAMP response element (CRE) in some MHC class II genes and phorbol ester response element (TRE) in others, overlaps the 3' end of the X box. Located upstream of the X box, the Z/W region contains a conserved S-box motif and a pyrimidine rich tract (PY). Induction of MHC class II genes by IFN-γ has been shown to require the cooperative action of all three of these regions (X, Y, and Z/W). Proteins that bind to these regions may themselves be targets for binding of other proteins which are IFN-γ inducible and do not contact DNA. Not all MHC class II promoters have TATA boxes. The alteration of the TATA box sequence has been shown to affect proper initiation of transcription only for the DRA gene. These
Figure 1.4. General features of the promoter proximal region (180 bp) of MHC class II genes (83). The start site and direction of transcription are shown by an arrow. Common regulatory elements (Y, X, X₂, and S) are found at similar locations in most MHC class II genes. PY is a pyrimidine rich region which lies directly upstream of the X box motif.
observations have led to the speculation that the X and Y boxes may in some way specify the transcriptional start site in MHC class II genes.

**Summary of results**

When this work was undertaken, the polymeric immunoglobulin receptor was under investigation mainly as a model protein for the study of transcytosis. pIgR is also of great importance as the precursor protein for secretory component, an essential part of the secretory immunoglobulin molecule. Regulation of the expression and release of pIgR as SC plays an important role in mucosal immune defense. The goal of this thesis was to study the mechanisms by which pIgR expression and release are regulated. This goal has been addressed in a number of ways.

Using extracellular calcium to control the polarity of HT-29 cells, the effects of cell polarity on pIgR expression and SC release were studied. Extracellular calcium modulated the release of SC from intestinal epithelial cells. Upon depolarization, an initial burst followed by a decline in SC release was observed. This decline in SC release was not accompanied by a decrease in cell-associated SC (pIgR).

Murine and human pIgR cDNAs have been isolated and sequenced, allowing recognition of the high degree of interspecies conservation of functional regions of the pIgR molecule. These
cDNAs have been used as tools for exploration of the mechanisms by which pIgR expression is regulated, including Northern analysis of the tissue specificity of murine pIgR expression and isolation of the promoter proximal region of the human pIgR gene.

To study the mechanism involved in regulation of pIgR expression by IFN-γ, a sensitive ribonuclease protection assay was developed and used to quantify pIgR mRNA in human intestinal epithelial cells. IFN-γ induced increases in pIgR protein were found to be accompanied by increases in steady state pIgR mRNA levels. The increase in pIgR mRNA was ablated by cycloheximide treatment, suggesting that regulation of pIgR by IFN-γ involves a mechanism requiring de novo protein synthesis.

To identify regulatory elements which may be involved in the control of pIgR gene expression, the 5'-flanking region of the human pIgR gene was isolated and sequenced. An analysis of the promoter proximal region of the gene has allowed the identification of several potential regulatory elements. Several of these motifs have been identified previously in the MHC class II genes which may explain apparent similarities in pIgR and MHC class II gene expression. Experiments addressing the significance of these elements in cytokine-responsive induction of pIgR expression in epithelial cells are already in progress in our laboratory.
CHAPTER 2

CELL POLARITY REGULATES THE RELEASE OF
SECRETORY COMPONENT, THE EPITHELIAL RECEPTOR
FOR POLYMERIC IMMUNOGLOBULINS, FROM THE
SURFACE OF HT-29 COLON CARCINOMA CELLS
Abstract

HT-29 human colon carcinoma cells can be induced to differentiate in glucose-free medium to an enterocytic phenotype characterized by intercellular tight junctions, a well organized brush border and expression of differentiation-specific proteins. We previously isolated a subclone, HT-29.74, which expresses high levels of secretory component, the epithelial receptor for polymeric immunoglobulins, when grown in glucose-free medium. We have used these cells as a model system for studying effects of cell polarity on SC expression and release from the cell surface. To study effects of cell polarity on SC release, differentiated HT-29.74 cells were depolarized by culturing in low calcium medium. Within two hours after transfer of the cells into low calcium medium, a burst of SC release was observed concomitant with cell depolarization. Subsequently, release of SC declined significantly and remained low as long as cells were maintained in a depolarized state. The extent of cell depolarization could be controlled by varying the extracellular calcium concentration or by substituting the divalent cation Sr²⁺, which partially prevents depolarization, for Ca²⁺. In either case, the magnitude of the initial burst and subsequent decline in release of SC was proportional to the extent of cell depolarization. Cell polarity appears to plays an important role in controlling the release of SC from intestinal epithelial cells, possibly by regulating the
distribution of membrane-bound SC and SC protease, which are on the basolateral and apical cell surfaces respectively, in differentiated cells.

Introduction

Secretory IgA, the predominant immunoglobulin in mucosal secretions, is the product of two cell types (6, 84). IgA-producing plasma cells in mucosal sites secrete dimeric IgA into the interstitial spaces of the lamina propria. A 100 kD epithelial membrane protein, membrane secretory component (mSC), acts as a specific receptor for the polymeric immunoglobulin and mediates its transport to the luminal surface. Studies of transcellular routing of SC in rabbit mammary gland (85), rat liver (33, 86-89) and Madin-Darby canine kidney (MDCK) epithelial cells transfected with rabbit SC cDNA (48), have led to the hypothesis that mSC is initially sorted to the basolateral membrane of polarized cells. Membrane SC on the basolateral surface is internalized by endocytosis, with or without its ligand, dIgA, after which the mSC-dIgA complex or mSC alone is transported across the epithelial cell to the apical surface. Proteolytic cleavage of mSC between the ectoplasmic and membrane-spanning domains results in release of an SC-dIgA complex called secretory IgA or an uncomplexed 80 kD molecule called free secretory component. The enzyme responsible for this cleavage and its specificity are unknown.
In rat hepatocytes, proteolytic cleavage of mSC from the cell surface is blocked by thiol protease inhibitors but is not inhibited by EDTA (34). Subcellular fractionation experiments designed to localize the protease activity (90) or the actual products of mSC cleavage (35), suggested that proteolytic cleavage occurs at the bile canalicular membrane, which is analogous to the apical (lumenal) surface of intestinal epithelial cells. The findings suggest that mSC is cleaved at the apical cell surface by a membrane-bound thiol protease that does not require divalent cation for activity. Access of apically localized protease to basolateral mSC is presumably restricted in polarized cells, preventing cleavage of mSC until it is transported to the apical surface.

The human colon carcinoma cell-line HT-29 (91) has been shown to express mSC and has been used to study IgA transport across cultured epithelial cells (12, 62, 92). However, immunofluorescence studies by our laboratory and others (64, 65, 92) indicate that less than 5% of uncloned HT-29 cells grown in glucose-containing medium express SC. HT-29 cells have been shown to differentiate under conditions of glucose deprivation to an enterocytic phenotype characterized by intercellular tight junctions, specialization of apical and basolateral domains, appearance of a well organized brush border and accumulation of specific apical membrane hydrolases (93). Louvard and coworkers have derived subclones of glucose-deprived HT-29
cells with differentiated phenotypes of either mucous or absorptive cells (94, 95).

The isolation of a series of HT-29 subclones that were selected for high levels of SC expression has been previously reported (63). The expression of SC by HT-29.74 cells was rapidly induced by glucose deprivation, followed by morphological changes consistent with increased enterocytic differentiation (64). To investigate the role of cell polarity in SC expression and release, differentiated HT-29.74 cells were depolarized by culturing in low calcium medium. The loss of cell polarity in low calcium medium was accompanied by a rapid initial increase in SC release, suggesting that reorganization of proteins in the plasma membrane may result in contact between basolaterally localized mSC and apically localized protease. With prolonged incubation in calcium deficient medium, depolarized HT-29.74 cells continued to express high levels of surface mSC but lost the ability to cleave it efficiently. Cell polarity apparently plays an important role in controlling the release of SC, possibly by regulating the distribution of membrane-bound SC and SC protease on the basolateral and apical cell surfaces.
Materials and Methods

Cell culture

The derivation of the HT-29.74 cell-line, which was selected for high plgR expression, has previously been described (64). HT-29.74 cells were induced to differentiate to an enterocytic phenotype by culturing in Leibovitz's L-15 medium (glucose-free) containing 5 mM galactose, 6 mM sodium pyruvate, 1 mM L-glutamine, 24 mM NaHCO₃, 20 mM HEPES, pH 7.2, 100 U/ml penicillin, 100 µg/ml streptomycin, 250 ng/ml amphotericin B, and 10% dialyzed FCS (64). For experiments comparing normal calcium (NCa) and low calcium (LCa) media, cells were grown in L-15 medium prepared from individual components with or without CaCl₂. To measure total calcium concentration, media samples were brought to a final concentration of 10% HNO₃ and 1 mM LaCl₃ and centrifuged; the resultant supernatants were analyzed for Ca²⁺ content with a Varian AA-575 atomic absorption spectrophotometer. The total concentrations of calcium were 1.03 mM in the normal calcium (NCa) medium and 50 µM in the low calcium (LCa) medium. LCa medium was supplemented as indicated with SrCl₂ or BaCl₂. To prevent precipitation of BaSO₄, sulfate-free LCa medium was used in Ba²⁺ supplementation experiments. All reagents for cell culture, including dialyzed FCS, were obtained from GibcoBRL (Grand Island, NY).
Enzyme-linked immunosorbent assay for SC

Levels of SC in culture supernatants and cell lysates were determined by ELISA as described (64). Microtiter plates were coated with a guinea pig antiserum to human SC (diluted 1:5000 (v/v) in PBS) for 1 hour at room temperature. The plates were washed with PBS containing 0.1% (w/v) BSA and residual sites were blocked with PBS containing 1% (w/v) BSA (PBS-BSA) for 1 hour at room temperature. The plates were then incubated for 1 hour at room temperature with either purified fSC, at concentrations ranging from 100 ng/ml to 0.1 ng/ml, or experimental samples. Purified fSC was diluted in culture medium for assay of culture supernatants or in cell lysis buffer (see below) for assay of cell lysates. Bound SC was detected by incubating with rabbit antiserum to human SC (diluted 1:2000 (v/v) in PBS-BSA containing 1% (v/v) normal guinea pig serum). Bound rabbit antibody was detected by incubating the plates with alkaline phosphatase-conjugated goat anti-rabbit IgG (Boehringer Mannheim Corp., Indianapolis, IN) diluted 1:1000 (v/v) in PBS-BSA. All incubations were for 1 hour at room temperature with three PBS-0.1% BSA washes after each step. Color was developed by adding disodium p-nitrophenyl phosphate (1 mg/ml w/v) in 50 mM glycine, 1 mM MgCl₂, pH 9.6. Absorbances were determined with a Dynatech MR 700 microplate reader at a wavelength of 410 nm. A third order regression equation with a range of 0.1-100 ng SC/ml was obtained, and concentrations of SC
in the experimental samples were extrapolated from the standard curve.

**Treatment of HT-29.74 cells with low calcium media**

HT-29.74 cells which had been maintained for 8 passages in glucose-free L-15 medium were plated at $1.5 \times 10^5$ cells/cm$^2$ in plastic multiwell dishes (area/culture $= 2$ cm$^2$) and grown to confluence before initiation of experimental treatments. Cells were then cultured for varying times in glucose-free, low calcium (LCa) medium (see above), with or without supplementation with Ca$^{++}$, Sr$^{++}$ or Ba$^{++}$, as described in the legends to Tables 2.1 and 2.2 and Figs. 2.1-2.4. Culture supernatants were collected and cells were lysed directly in the dishes with cell lysis buffer (0.1 M sodium phosphate, pH 7.5, 1% (v/v) Tween-80, 0.5 M phenyl methyl sulfonyl flouride, 5 mM EDTA, 0.12% (v/v) aprotinin and 10% (v/v) fetal calf serum). Cells in parallel cultures were detached with 0.05% (w/v) trypsin and 0.53 mM EDTA in Hanks' balanced salt solution and counted. Concentrations of SC in culture supernatants and cell lysates were determined by ELISA as described above. For determination of % trypsin sensitive SC, replicate cultures of intact cells were treated with 0.25% trypsin (Sigma Chemical Co., St. Louis, MO) in Dulbecco's phosphate buffered saline with or without CaCl$_2$ for 10 minutes (min.). at 37°C, and then lysed as above. Trypsin sensitive SC was defined as: (total cell associated SC) minus (trypsin insensitive SC). All
experiments were repeated at least 3 times. Data were analyzed by multiple analysis of variance, and group means were compared post hoc by the method of least significant difference.

**Determination of cytosolic free calcium**

Confluent monolayers of HT-29.74 cells were grown on coverslips (14 x 16 mm) made of Aclar plastic (Allied Engineered Plastics, Pottsville, PA). For 24 hours before the assay, individual monolayers were cultured in either NCa or LCa medium. Cells were loaded for 45 min. at 37°C with fura-2 acetoxymethyl ester (fura-2 AM, Molecular Probes, Eugene, OR) dissolved in DMSO and added to a concentration of 2 μM in NCa or LCa medium devoid of amino acids, vitamins and phenol red. Monolayers were then washed and incubated in loading medium without fura-2 AM for an additional 10 min. to allow hydrolysis of the entrapped ester. After incubation, the cultures were washed in a balanced salt solution containing 25 mM HEPES, pH 7.4, 120 mM NaCl, 5 mM KCl, 1.5 mM MgCl₂, 0.1% (w/v) bovine serum albumin, 5 mM galactose and 5 mM pyruvate, with or without 1.03 mM CaCl₂. Fluorescence of the fura-2 loaded monolayers was measured in a fluorescence spectrophotometer (96) with excitation and emission wavelengths of 339 and 500 nm respectively. Determinations were performed at 37°C with constant mixing. To observe possible fluxes in cytosolic free calcium during changes in extracellular calcium concentrations, cytosolic free calcium levels were measured
following addition of 1.03 mM EGTA to monolayers cultured in NCa medium, and following addition of 1.03 mM CaCl$_2$ to monolayers cultured in LCa medium. Maximal fura-2 fluorescence was determined by treatment of the cells with 10 μM ionomycin (Calbiochem, La Jolla, CA) in the presence of extracellular calcium. Minimal fluorescence was determined by addition of 100 μM MnCl$_2$. Ca$^{++}$ dependent fluorescence was 70% of the Mn$^{++}$ quenchable fluorescence. The concentration of intracellular calcium was calculated from the following equation, using 224 nM as the apparent K$_d$ of fura-2 for Ca$^{++}$ (97):

$$[\text{Ca}^{++}]_i = 224 \text{ nM} \frac{(F - F_{\text{min}})}{(F_{\text{max}} - F)}.$$  

Results

Culture of HT-29.74 cells in low calcium medium disrupts cell polarity

We hypothesized that loss of cell polarity might disrupt the segregation of apically localized protease activity from basolaterally localized SC, leading to a transient increase in release of SC from the cell surface. With prolonged loss of cell polarity, defects in transport and/or sorting of either SC or protease to the cell surface could lead to decreased release of SC. To test this hypothesis, differentiated HT-29.74 cells were induced to depolarize by culturing in low calcium medium, a treatment known to disrupt polarity of cultured epithelial cells (98). HT-29.74 cells which had been maintained for 8 passages in glucose-
free medium were grown to confluence in glucose-free medium containing 1.03 mM Ca$^{++}$ (NCa) and transferred to glucose-free medium containing 50 μM Ca$^{++}$ (LCa). HT-29.74 cells transferred to LCa medium began to round up within 2 hours (Fig. 2.1D), and by 24 hours exhibited marked changes in cell shape characteristic of loss of cell polarity and intercellular contacts (Fig. 2.1G). To induce partial depolarization, confluent cultures of HT-29.74 cells were transferred to LCa medium supplemented with 100 μM CaCl$_2$ or 1.89 mM Sr$^{++}$, a divalent cation which can substitute partially for Ca$^{++}$ in maintaining intercellular junctions (99). Addition of 100 μM CaCl$_2$ to the LCa medium partially inhibited the loss of cell polarity; no change in cell shape was observed after 2 hours (Fig. 2.1E), and after 24 hours the cells were significantly less rounded than those in LCa medium (Fig. 2.1H). In cells cultured with 1.89 mM Sr$^{++}$, the degree of rounding at 2 hours was similar to that seen in LCa medium (Fig. 2.1F). However, after 24 hours an inhibition of further depolarization was observed, resembling that seen in cultures supplemented with 100 μM CaCl$_2$ (Fig. 2.1I). When LCa medium was supplemented with another divalent cation, Ba$^{++}$, no inhibition of cell rounding was observed (data not shown). HT-29.74 cell polarity under the various media conditions used in this experiment was confirmed by electron microscopy (64).

Levels of cytosolic free calcium were monitored to determine whether changes in extracellular calcium caused
Figure 2.1. Transfer of HT-29.74 cells into low calcium medium results in changes in cell shape consistent with loss of cell polarity. HT-29.74 cells were grown to confluence in glucose-free, normal calcium medium, then transferred to glucose-free, low calcium medium without supplementation (A, D, G) or supplemented with 100 μM CaCl₂ (B, E, H) or 1.89 mM SrCl₂ (C, F, I). A-C, immediately after transfer to fresh medium; D-F, the same fields shown in panels A-C, 2 hours after transfer; G-I, different fields from the same cultures shown in A-C, 24 hours after transfer. Magnification, 100x.
changes in intracellular calcium concentration. No fluxes in cytosolic free Ca\textsuperscript{++} concentration were observed immediately following addition of 1.03 mM EGTA to cells in NCa medium or addition of 1.03 mM CaCl\textsubscript{2} to cells in LCa medium (data not shown). After 24 hours in LCa medium, the cytosolic free Ca\textsuperscript{++} concentration was 30 nM (n = 2) compared to 57 nM (n = 2) for cells in NCa medium. While some gradual depletion of cytosolic free calcium occurs when HT-29.74 cells are cultured in calcium-deficient medium, it is insufficient to explain the rapid changes that were observed in release of fSC (see below), which are more likely related to changes in cell polarity.

**Disruption of cell polarity is accompanied by changes in release of SC from the cell surface**

To determine the effect of loss of cell polarity on release of SC, confluent cultures of HT-29.74 cells were transferred from NCa to LCa medium, and release of SC into the cell supernatant was monitored over a 24-hour period (Fig. 2.2). A marked increase in release of SC was observed within 2 hours following transfer into LCa medium; very little additional SC was released in the ensuing 22 hours. In contrast, cells maintained in NCa medium continued to release SC at a constant rate. The burst of SC release accompanying the visible rounding up of cells in LCa medium is consistent with the hypothesis that basolaterally localized SC had intermixed with apically localized protease. When HT-29.74 cells
Figure 2.2. Depolarization of HT-29.74 cells in low calcium medium is accompanied by a burst in release of SC. HT-29.74 cells were grown to confluence in glucose-free, normal calcium medium, then transferred either to glucose-free, low calcium medium (open symbols) or fresh normal calcium medium (closed symbols). At the indicated times after transfer, culture supernatants were removed and assayed for SC. Results are presented as cumulative SC levels (mean ± S.E.M., n = 3); asterisks denote groups where the mean for cells cultured in low calcium medium is significantly greater than the mean for cells cultured in normal calcium medium (p < .01).
in LCa medium were transferred back into NCa medium for 24 hours, cells repolarized and the rate of release of SC returned to normal levels (data not shown).

To test whether prolonged cell depolarization affected the expression as well as the release of SC, levels of cell-associated and released SC were compared in HT-29.74 cells cultured for 1 or 5 days in LCa medium (Table 2.1). No difference in release of SC was observed during the first day in LCa medium (compare groups 1 and 2), consistent with the observation that a large initial burst of SC release occurs only immediately following loss of cell polarity (Fig. 2.2). However, after 5 days in LCa medium a significant decrease in release of SC was observed (group 3). No significant reduction was observed in the pool of trypsin-sensitive (presumably cell-surface) SC, suggesting that the deficit in release of SC was related to the rate of proteolytic cleavage rather than impaired transport of SC to the cell surface. The decrease in release of SC was balanced by a corresponding increase in cell-associated SC, suggesting that prolonged depolarization of HT-29.74 cells did not diminish production of SC.

To strengthen the hypothesis that changes in cell polarity affect release of SC, HT-29.74 cells were cultured for 24 hours in varying concentrations of extracellular calcium (Fig. 2.3). Culture supernatants were collected after 2 hours (to determine if a burst of SC release had occurred), then fresh medium was added for the final 22 hours. At Ca$$^{++}$$ concentrations of less than 100 µM, the
<table>
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<th>Group</th>
<th>1</th>
<th>2</th>
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<tbody>
<tr>
<td>Medium</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Days 1-4</td>
<td>NCa</td>
<td>NCa</td>
<td>LCa</td>
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<tr>
<td>Day 5</td>
<td>NCa</td>
<td>LCa</td>
<td>LCa</td>
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<tr>
<td>Released SC</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>(ng/10^6 cells/24 hours)</td>
<td>24.0 ± 1.04</td>
<td>23.5 ± 3.06</td>
<td>15.8 ± 3.14^2</td>
</tr>
<tr>
<td>Cell-associated SC</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Total (ng/10^6 cells)</td>
<td>61.1 ± 5.22</td>
<td>54.1 ± 4.54</td>
<td>86.3 ± 6.49^2</td>
</tr>
<tr>
<td>% Trypsin sensitive</td>
<td>27.2 ± 4.78</td>
<td>8.0 ± 8.59</td>
<td>21.8 ± 2.50</td>
</tr>
<tr>
<td>Released/cell-associated</td>
<td>0.40 ± 0.025</td>
<td>0.43 ± 0.021</td>
<td>0.19 ± 0.042^2</td>
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^1 HT-29.74 cells were grown to confluence in glucose-free, normal calcium (NCa) medium. Group 1 cells were cultured in NCa medium for all 5 days of the experiment. Group 2 cells were cultured in NCa medium for 4 days, then transferred to glucose-free, low calcium (LCa) medium for the final day of the experiment. Group 3 cells were cultured in LCa medium for all 5 days of the experiment. All cultures were harvested on day 5 to ensure that they were at the same stage of confluence. Culture supernatants and cell lysates from the last 24 hours of the experiment were assayed for SC. Data are expressed as mean ± S.E.M. (n = 4).

^2 Mean is significantly different from the corresponding mean for group 1 (p < .05).
Figure 2.3. Effect of extracellular calcium concentration on SC release. HT-29.74 cells were grown to confluence in glucose-free, normal calcium medium, then transferred to medium containing 50-1030 μM Ca++. After 2 hours, culture supernatants were removed and cells were cultured with fresh medium for an additional 22 hours. Culture supernatants were assayed for SC. (A), SC released during hours 0-2; average of 2 experiments (n = 6). (B), SC released during hours 2-24; average of 3 experiments (n = 10). Results are presented as mean ± S.E.M. Asterisks denote groups where the mean is significantly different from the mean for cells grown in low calcium medium (50 μM Ca++) (p < .05).
initial burst of SC release was observed, followed by a significant decrease in release of SC over the subsequent 22 hours; the magnitude of these responses was proportional to the extracellular concentration of Ca++. The observation that SC release was relatively normal at concentrations of Ca++ above 100 μM is consistent with the morphological data demonstrating that this level of Ca++ partially inhibits changes in cell shape (Fig. 2.1).

**Effect of Ca++ analogs on release of SC**

The effect of supplementation of LCa medium with 1.89 mM Sr++ (which partially inhibits loss of cell polarity; Fig. 2.1I) or 1.26 mM Ba++ (which does not inhibit loss of cell polarity) on release of SC were also tested (Table 2.2). Neither Sr++ nor Ba++ prevented the burst of release of SC during the initial 2 hours of incubation in LCa medium. However, supplementation of LCa medium with Sr++, but not Ba++, prevented the decline in release of SC seen during the subsequent 22 hours of incubation in LCa medium. Because it was necessary to remove sulfate from the Ba++ supplemented medium (to prevent precipitation of BaSO₄), control groups cultured in sulfate-free NCa and LCa media were included (Table 2.2). No effect on SC release was observed due to removal of sulfate from the media. To test the dose dependence of the Sr++ effect, HT-29.74 cells were cultured for 24 hours in LCa medium supplemented with varying concentrations of Sr++ ranging from 0.3 - 3.4 mM. No significant effect of Sr++ at any concentration
<table>
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<tr>
<th>Medium</th>
<th>Released SC (ng/10^6 cells)</th>
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<tbody>
<tr>
<td></td>
<td>0-2 hrs.</td>
</tr>
<tr>
<td>NCa</td>
<td>0.9 ± 0.12a</td>
</tr>
<tr>
<td>LCa</td>
<td>2.9 ± 0.16c</td>
</tr>
<tr>
<td>LCa + Sr++</td>
<td>2.1 ± 0.23b</td>
</tr>
<tr>
<td>NCa - SO₄</td>
<td>0.9 ± 0.08a</td>
</tr>
<tr>
<td>LCa - SO₄</td>
<td>2.7 ± 0.19b,c</td>
</tr>
<tr>
<td>LCa - SO₄ + Ba++</td>
<td>2.9 ± 0.07c</td>
</tr>
</tbody>
</table>

1HT-29.74 cells were grown to confluence in glucose-free, normal calcium (NCa) medium, then transferred to one of the following media: fresh NCa; glucose-free, low calcium (LCa) medium; LCa supplemented with 1.89 mM SrCl₂; sulfate-free NCa; sulfate-free LCa; or sulfate-free LCa supplemented with 1.26 mM BaCl₂. Ba⁺⁺ supplemented medium was prepared without sulfate to prevent precipitation of BaSO₄. After 2 hours, culture supernatants were removed and cells were cultured with fresh medium for an additional 22 hours. Culture supernatants were assayed for SC. Data are expressed as mean ± S.E.M. (n = 4).

a,b,cMeans within each column sharing a superscript are not statistically different (p > .05).
was observed during the first 2 hours; the initial burst of release of SC was similar to that seen in cultures given LCa medium alone (Fig. 2.4A). In contrast, the magnitude of release of SC in the subsequent 22 hours was proportional to the dose of Sr\(^{++}\), and at concentrations of 1 mM or more was significantly greater than that observed with LCa medium alone (Fig. 2.4B). These data are consistent with the observed changes in cell shape: cells cultured in 1.89 mM SrCl\(_2\) began to round up after 2 hours (Fig. 2.1F), concomitant with the burst of SC release; after 24 hours depolarization was only partial (Fig. 2.1I), consistent with an intermediate level of release of SC. These observations suggest that maintenance of cell polarity is required for normal release of SC in intestinal epithelial cells.

**Discussion**

HT-29.74 cells, which had been induced to differentiate by long-term glucose deprivation, were used as a model to study the polarized expression and release of SC. In rat hepatocytes, polarity of SC release is thought to be maintained by segregation of mSC to the basolateral (sinusoidal) surface and SC protease to the apical (bile canalicular) surface (35, 90). Release of fSC, alone or bound to polymeric immunoglobulin, into bile should therefore occur only when mSC is transcytosed to the bile canaliculus. Depolarization of HT-29.74 cells might disrupt the regulated release of fSC from the cell surface. To test this hypothesis, HT-
Figure 2.4. Addition of Sr$^{++}$ to low calcium medium supports SC release. HT-29.74 cells were grown to confluence in glucose-free, normal calcium medium, then transferred to glucose-free, low calcium medium supplemented with 0-3400 μM SrCl$_2$. After 2 hours, culture supernatants were removed and cultures were cultured with fresh Sr$^{++}$ supplemented medium for an additional 22 hours. Culture supernatants were assayed for SC. A. SC released during hours 0-2; average of 2 experiments (n = 5). B. SC released during hours 2-24; average of 3 experiments (n = 10). Results are presented as mean ± S.E.M. Asterisks denote groups where the mean is greater than the mean for cells grown in low calcium medium (p < .05). Open circles indicate SC release by parallel cultures grown in normal calcium medium.
29.74 cells were cultured in low calcium medium, a treatment which disrupts cell-cell contacts and leads to loss of apical/basolateral polarity in cultured epithelial cells (98). The loss of cell polarity was accompanied by a rapid initial increase, followed by a significant inhibition of release of SC from the cell surface (Fig. 2.2). No immediate changes in cytosolic free Ca\textsuperscript{++} concentration were observed following removal of extracellular Ca\textsuperscript{++}, suggesting that the changes in SC release were secondary to loss of cell polarity and were not signaled by intracellular Ca\textsuperscript{++} fluxes. Supplementation of low calcium medium with Sr\textsuperscript{++}, a divalent cation which has been shown to preserve polarity of embryonal carcinoma cells in low calcium medium (99), was accompanied by partial prevention of both the loss of polarity of HT-29.74 cells and the inhibition of SC release (Fig. 2.1 and Table 2.2). Ba\textsuperscript{++}, which does not support polarity of embryonal carcinoma cells (99), had no effect on either cell polarity or SC release in HT-29.74 cells cultured in low calcium medium. Our observations in HT-29.74 cells are consistent with the hypothesis that loss of cell polarity may result in the intermixing of basolaterally localized SC with apically localized SC protease.

Prolonged culture in low calcium medium could result in an inhibition of transport of either mSC or SC protease to the cell surface, preventing normal cleavage and release of fSC. However, no decline in cell surface SC following prolonged culture of HT-29.74 cells in low calcium medium was observed (Table 2.1).
has been reported that depolarized epithelial cells exhibit reduced transport of apical, but not basolateral, membrane proteins to the cell surface (98, 100). Therefore, the hypothesis that long-term inhibition of SC release in depolarized HT-29.74 cells results from inhibition of transport of SC protease to the cell surface is favored.

In conclusion, HT-29.74 cells provide a unique model for studying the regulation of expression and release of SC. Our results demonstrating a role for cell polarity in SC release are consistent with the hypothesis that cleavage of mSC to fSC occurs on the apical surface of intestinal epithelial cells.
CHAPTER 3

MOLECULAR CLONING OF MOUSE AND HUMAN POLYMERIC IMMUNOGLOBULIN RECEPTOR cDNAs
Abstract

Transcytosis of polymeric immunoglobulin and IgA immune complexes by mucosal epithelial cells is mediated by the polymeric immunoglobulin receptor. This study describes the isolation of cDNAs which span the complete coding sequences of mouse and human pIgR. The 3077 bp murine pIgR cDNA contains 2313 nucleotides of coding sequences, 84 nucleotides of 5'-untranslated, and 679 nucleotides of 3'-untranslated sequences. The human pIgR cDNA sequence (2918 bp) includes a coding region of 2295 nucleotides, a 5'-untranslated region of 180 nucleotides and a 3'-untranslated region of 443 nucleotides. Northern blot analysis reveals a murine pIgR transcript of approximately 3.9 kb expressed in liver and intestinal epithelial cells. A comparison of the predicted amino acid sequences of mouse, human, rat and rabbit pIgR demonstrates that both the cytoplasmic region, which contains signals for intracellular trafficking, and the transmembrane region are very homologous across species. Although inter-species sequence homology appears to be lowest in the extracellular region, this homology is strikingly clustered in the amino-terminal domain which is thought to be involved in the initial noncovalent association of pIgR with IgA. In conclusion, regions of the pIgR molecule implicated in the functions of IgA binding and epithelial transcytosis appear to have been preferentially conserved.
Introduction

Secretory immunoglobulin (sIg) provides an effective immune barrier at mucosal sites by blocking entry of antigens and neutralizing microbial pathogens (16, 17). The polymeric immunoglobulin receptor, an integral membrane glycoprotein, transports the polymeric immunoglobulins, IgA and IgM, into external secretions (12, 17, 31). PIgR has also been shown to bind and transport polymeric IgA (pIgA) immune complexes (22, 101). Polymeric immunoglobulin is bound by the pIgR at the basolateral (and sinusoidal, in rodent hepatocytes) surface of many types of glandular epithelial cells. The receptor-ligand complex then undergoes endocytosis, followed by transcytosis to the apical (and bile canalicular, in rodent hepatocytes) cell surface and a cleavage event which releases the sIg complex from the cell. Secretory component, a glycoprotein composed of the extracellular domains of the receptor, becomes an integral part of the sIg molecule (102). SC has been proposed to protect sIg from proteolytic degradation and denaturation in the mucosal environment (13-15).

The structure and function of rabbit pIgR have been studied extensively (39). Polymeric immunoglobulin binds to the extracellular region of the receptor, composed of five immunoglobulin-like domains and a sixth more distantly related domain. The initial noncovalent interaction of the receptor with pIg has been shown to involve the amino-terminal extracellular
domain of PIgR (45). The large cytoplasmic region of the receptor has been shown to contain signals important in basolateral targeting and endocytosis (48-50). Several different phosphorylated forms of PIgR have been shown to exist, and it has been suggested that phosphorylation of the cytoplasmic region of PIgR plays a role in receptor trafficking (51, 52).

Full length cDNA sequences for both rabbit (39) and rat PIgR (40) have been previously reported. A partial human cDNA for PIgR has been reported which lacks the 5'-untranslated, leader peptide and N-terminal PIg-binding domain sequences (41). The purpose of this study was to isolate mouse and human PIgR cDNAs containing 5'-untranslated and full length coding sequences, to analyze PIgR inter-species sequence homology in functionally important regions of the molecule, and to examine the tissue specificity of murine PIgR expression. Availability of cDNAs for mouse and human PIgR would provide probes for investigation of mechanisms of PIgR expression, including isolation of the promoter regions of the mouse and human PIgR genes. In this study, the isolation of a 3077 bp cDNA from mouse liver and a 2918 bp cDNA from human breast that contain the complete coding region of PIgR are described. Alignments of the nucleotide sequences of cDNAs from mouse, human, rat (40) and rabbit (39) revealed that homology is significant, especially within the coding region. Analysis of the predicted amino acid sequences of mouse, human, rat and rabbit PIgR shows that regions of the molecule
implicated in pIg binding and transcytosis have been highly conserved across species. In addition, a 3.9 kb mRNA for pIgR is demonstrated which is expressed in murine liver and intestinal epithelial cells.

Materials and Methods
Cloning and sequencing of mouse and human pIgR cDNAs

Approximately 500,000 recombinants from a mouse liver cDNA library, constructed in the lambda ZAP vector (Catalog #935302, Stratagene, La Jolla, CA), were screened using a cDNA probe spanning nucleotides -21 to 957 of the rat polymeric immunoglobulin receptor cDNA (40). The probe was simultaneously amplified and radiolabeled by performing the polymerase chain reaction (PCR) (Perkin-Elmer Cetus, Norwalk, CT) in the presence of $[^{32}P]$-dCTP (3000 Ci/mmol; NEN Research Products, Boston, MA) without the addition of unlabeled dCTP (103). The final concentrations of the reactants in the 100 μl reaction mixture were as follows: 10 mM Tris-HCl (pH 8.3); 50 mM KCl; 1.5 mM MgCl$_2$; 0.001% (w/v) gelatin; 20 μM each dATP, dGTP, dTTP; 0.5 μM $[^{32}P]$-dCTP; 0.7 pM DNA template; 0.1 μM each primer. After the addition of 2.5 U of Taq polymerase (Perkin Elmer Cetus), amplification was performed using a DNA thermal cycler (Perkin Elmer Cetus) for 40 cycles of 1 min. at 94°C, 2.5 min. at 55°C and 4 min. at 72°C; followed by a final elongation for
10 min. at 72°C. The 5'-sense primer that was used for the PCR had the sequence 5' ACG TAC AAG CTT CCT CCT TGG AAG CCA CAA GCG A 3', and the 3'-antisense primer had the sequence 5' TGC ATC CTC CTT CCT CAG GCC TG 3'. The rat pIgR cDNA was used as template for the PCR.

The library was screened by infecting BB4 host bacteria with 25-50,000 plaque forming units (104, 105), and plating in 0.7% (w/v) agarose (GibcoBRL) on 150 mm NZY agar plates (0.5% (w/v) NaCl, 0.2% MgSO₄·7H₂O, 0.5% bacto-yeast extract, 1% bacto-casamino acids and 1.5% bacto-agar, DIFCO, Detroit, MI). Plates were incubated at 37°C for 16 h. Plaques were 1-3 mm in size. To prevent sticking, plates were chilled at 4°C for 1 h before duplicate lifts were taken using nitrocellulose filters (HATF, Millipore Corp., Marlborough, MA). The first lift was done for 5 min., and the second lift was done for 10 min. Adherent DNA was denatured in 1.5 M NaCl, 0.5 N NaOH for 2 min., neutralized in 1.5 M NaCl, 0.5 M Tris-HCl (pH 8.0) for 5 min., and rinsed in 2X SSC, 0.2 M Tris-HCl (pH 7.5) (106).

Filters were baked overnight at 42°C, prehybridized for 2 h at 42°C in 50% (v/v) formamide, 4X SSC, 1X Denhart's solution, 10% (w/v) dextran sulfate (MW 300,000), 0.1% (w/v) SDS, 0.02 M Tris-HCl (pH 7.5) and 50 μg/ml of denatured salmon sperm DNA, and then hybridized for 18 h at 42°C in the same solution with 1 x 10⁶ cpm of denatured ³²P-labeled probe added. Filters were washed 2X in 2X SSC, 0.1% SDS at 22°C for 10 min.; then 2X in 0.2X
SSC, 0.1% SDS at 65°C for 30 min. Filters were exposed to Kodak XAR-5 film at -70°C using intensifying screens.

Thirty hybridizing clones were identified. Five of these clones were purified (107) and excised as pBluescript (SK-) subclones by the addition of helper phage (Exassist, Stratagene) (108). The 5' and 3' ends of cDNAs were sequenced by the dideoxy chain-termination method (109) using the modified T7 DNA polymerase enzyme (110), Sequenase (United States Biochemical Corporation., Cleveland, OH), and primers corresponding to sequences within the T3 and T7 RNA polymerase promoter regions of the pBluescript vector. All five contained mouse pIgR cDNA as determined by sequence comparisons with rat pIgR (40). One cDNA, containing the complete coding sequence of mouse pIgR, was sequenced using additional sequence-specific primers to obtain the complete sequences from both strands (Fig. 3.1).

To obtain a cDNA containing the 5' end of the human pIgR mRNA, both genomic DNA and cDNA clones encoding human pIgR were isolated. Over one million recombinants from a λDASH human lymphocyte genomic library (Catalog #943202, Stratagene) were screened as described above, using the LE-392 bacterial host strain and a PCR-generated human pIgR cDNA probe spanning 183 nt from the 5' end of the partial human pIgR cDNA sequence reported by Krajci et al. (41). Synthetic oligonucleotides used as primers in the PCR corresponded to nucleotides 214-243 and the
reverse complement of nucleotides 372-396 of the human pIgR coding sequence (Fig. 3.2). Template cDNA for the PCR was a 612-bp cDNA fragment spanning nucleotides 214-825 of the human pIgR coding sequence that was itself generated by PCR (GeneAmp® PCR reagent kit, Perkin Elmer Cetus). The final concentrations of the reactants in the 100 µl PCR mixture were as follows: 10 mM Tris-HCl (pH 8.3); 50 mM KCl; 1.5 mM MgCl₂; 0.001% (w/v) gelatin; 200 µM each dATP, dGTP, dTTP and dCTP; 1 µM each primer; 2.5 U of Taq polymerase and 5 µg of template cDNA/ 100 µl. The amplification cycle is described above. The 5'-sense primer had the sequence 5' GGC TAC GTC TCC AGC AAA TAT GCA GGC AGG 3', and the 3'-antisense primer had the sequence 5' ACT GCT CTG TCG GCA CAG AAA TTT GGC CAC 3'. Template cDNA for this PCR was prepared by reverse transcription of poly A+ RNA isolated from HT-29.74 cells, using Moloney murine leukemia virus reverse transcriptase (GibcoBRL) and oligo dT as primer.

One human pIgR genomic clone was identified. λDNA was prepared from this clone by PEG precipitation (111) and digested with the enzyme, Bgl II (GibcoBRL). After electrophoresis in 0.5% agarose, the DNA was depurinated in 2.5 N HCl, denatured in 1.5 M NaCl, 0.5 N NaOH and transferred to Genescreen® (NEN Research Products) by capillary blotting. The filter was baked at 90°C for 2 hours, and then prehybridized, hybridized (with the PCR-generated human pIgR cDNA probe described above) and washed under the same conditions used to screen the genomic library.
Southern blot analysis (data not shown) resulted in identification of a 1.5 kb-hybridizing fragment which was subcloned into pBluescript and sequenced as described above, using as sequencing primers the same primers that were used to generate the probe. By comparing the predicted amino acid sequence of this fragment with the sequence of human SC (38), an exon containing the N-terminus of the human pIgR protein was located. The sequence of this exon was used to design a probe for screening a human cDNA library.

Seven human pIgR cDNA clones were isolated as described above from a λgt11 human breast cDNA library (Clontech Laboratories, Pala Alto, CA) using the LE-392 bacterial host strain and a PCR-generated cDNA probe spanning nucleotides 58-209 of the human pIgR coding sequence. The oligonucleotide primers used in the PCR corresponded to nucleotides 58-81 and the reverse complement of nucleotides 186-209 (Fig. 3.2). The human pIgR genomic DNA was used as template for the PCR. Four of the resultant cDNA clones were subcloned into the pBluescript II (SK-) plasmid (Stratagene) and sequenced as described using both human pIgR sequence-specific primers and primers corresponding to sequences in the pBluescript vector. The identity of the cDNA clones was verified by comparison with the partial nucleotide sequence of human pIgR reported by Krajci et al. (41) and the protein sequence of human SC (38). Two cDNA clones (hpIgR-1
and hpIgR-2) were found to span the complete coding sequence of human pIgR (Fig. 3.2).

Comparisons between nucleotide and amino acid sequences of mouse, rat (40), human and rabbit (39) pIgR cDNAs were made using the ALIGN and AALIGN programs (DNASTAR, Inc., Madison, WI). Nucleotide sequence alignments were made separately for the 5′-untranslated regions (84, 73, 180, and 123 nucleotides (nt) for mouse, rat, human and rabbit cDNAs, respectively); coding regions (2313, 2310, 2295, and 2322 nt for mouse, rat, human and rabbit cDNAs, respectively); and 3′-untranslated regions (679, 703, 443, 1075 nt for mouse, rat, human and rabbit cDNAs, respectively).

**Isolation of mouse intestinal epithelial cells**

After removal of Peyer's patches, mouse small intestines were slit, rinsed in Hank's balanced salt solution (HBSS), cut into 2 cm pieces and incubated twice in 0.05% (w/v) trypsin, 0.53 mM EDTA (GibcoBRL) at 37°C for 30 minutes. The cell suspensions were pelleted, incubated at 37°C for 10 min. in 5 ml of HBSS containing 3 mg/ml pronase (Calbiochem, San Diego, CA) and 0.3 mg/ml DNase (Sigma). After washing and resuspending the cells in 5 ml of calcium and magnesium-free HBSS (CaMg-free HBSS), the epithelial cells were purified by density gradient centrifugation for 20 min. at 22°C and 17,000g, using 33% (v/v) Percoll (Pharmacia LKB Biotechnology Inc., Piscataway, NJ).
Epithelial cells were collected from the top of the Percoll layer, and washed twice in CaMg-free HBSS. Trypan blue staining demonstrated that 95% of the recovered epithelial cells were viable.

**Northern blot analysis**

Total cellular RNA was isolated from mouse liver, spleen and epithelial cells by a modification of the technique of Chomczynski and Sacchi (112). Briefly, tissues were homogenized or epithelial cells were lysed in RNAzoI™ (Cinna/Bio-tecx, Friendswood, TX), 0.1 volume of chloroform was added, and the mixture was shaken, followed by incubation on ice for 15 min. After centrifugation at 4°C for 20 min. at 12,000g, the aqueous phase was collected and precipitated overnight at -20°C by the addition of an equal volume of isopropanol. RNA was pelleted by centrifugation at 4°C for 30 min. at 12,000g, and denatured with glyoxal and dimethyl sulfoxide before being size-fractionated by electrophoresis in 1% agarose. RNA was then transferred to Genescreen® (NEN Research Products) by capillary blotting and the filter was baked at 80°C for 3 hours. The blot was prehybridized for 3 h at 42°C in 50% formamide, 5X SSPE, 5X Denhart's solution, 5% (w/v) dextran sulfate and 100 μg/ml boiled salmon sperm DNA. Hybridization was performed at 42°C for 18 h in the same solution as the prehybridization with ³²P-labeled mouse actin and pIgR probes added, but without heterologous DNA. The actin probe was
prepared by random priming of a 1.15 kb Pst I cDNA fragment containing mouse actin coding sequence. The pIgR cDNA probe, corresponding to nucleotides 431-770 of the mouse pIgR coding sequence (see Fig. 3.1), was generated by PCR using as template the mouse pIgR cDNA (described above). Primers used in the PCR corresponded to nucleotides 431-470 and the reverse complement of nucleotides 744-770 of the mouse pIgR coding sequence. The blot was washed in 0.2X SSPE, 0.1% SDS at 55°C and visualized by autoradiography using an intensifying screen and Kodak XAR-5 film.

Results

Nucleotide sequences of mouse and human pIgR cDNAs

Screening of a mouse liver cDNA library with a rat pIgR cDNA probe led to the isolation of more than thirty putative mouse pIgR cDNAs. Five cDNAs were excised, sequenced and found to contain sequences homologous to rat pIgR (40). One of these cDNAs contains the entire coding sequence of mouse pIgR, 84 nucleotides of 5'-untranslated and 677 nucleotides of 3'-untranslated region sequences (Fig. 3.1).

One genomic DNA clone and seven cDNA clones encoding human pIgR mRNA were isolated and sequenced. Analysis of two overlapping cDNA clones, hpIgR-1 and hpIgR-2, yielded the nucleotide sequence of the complete coding region of human pIgR mRNA, as well as 180 nt of 5'-UTR and 443 nt of 3'-UTR.
Fig. 3.1. Nucleotide sequence of mouse polymeric immunoglobulin receptor cDNA. Five overlapping cDNA clones encoding mouse pIgR were isolated from a mouse liver cDNA library and sequenced. By sequence comparison with rat pIgR (40), one of the clones was found to contain the complete coding sequence of mouse pIgR (2313 nt), 84 nucleotides of 5'-untranslated and 677 nucleotides of 3'-untranslated sequences. Numbering is relative to the start site of translation (position +1); negative numbers are given to the 5'-untranslated region. The deduced amino acid sequence for mouse pIgR obtained from this clone is also shown. The amino acids are given below the nucleotide sequence in three-letter code. The N-terminal lys residue of the mature protein (amino acid 19 of the primary translation product) is underlined.
sequences (Fig. 3.2). The coding sequence for human pIgR was identical to that reported by Krajci et al. (41) except at position 956, where a C residue in contrast to A was observed; this difference in the nucleotide sequence did not, however, change the Arg residue encoded at this position.

Alignments of the nucleotide sequence of mouse pIgR cDNA (Fig. 3.1) with the corresponding sequences from rat (40), human (Fig. 3.2) and rabbit (39) revealed that homology was high within the coding region (90% nucleotide sequence identity for mouse vs. rat, 77% for mouse vs. human, 68% for mouse vs. rabbit). Interestingly, the homology at the level of amino acid sequence was less than at the level of nucleotide sequence (86% amino acid sequence identity for mouse vs. rat, 65% for mouse vs. human, 49% for mouse vs. rabbit). While considerable homology (68%) was noted between the human and rabbit pIgR cDNAs in the 5'- and 3'-UTR sequences, considerable divergence was observed in the rat sequences, particularly in the 5'-UTR. However, the mouse and rat 5'-untranslated sequences exhibit much greater similarity (59%).

**Functional regions of the pIgR protein are highly conserved across species**

The mouse and human pIgR cDNAs encode proteins of 771 and 824 amino acids, respectively. An alignment of the predicted amino acid sequences of mouse (Fig. 3.1), rat (40), human (Fig.
Figure 3.2. Nucleotide sequence of human polymeric immunoglobulin receptor cDNA. A. Two overlapping cDNA clones encoding human pIgR mRNA (hpIgR-1 and hpIgR-2) were isolated from a human breast cDNA library and sequenced. In addition to the complete coding sequence (2295 nt; shaded bar), these cDNAs encoded 180 and 443 nucleotides respectively of the 5'- and 3'-untranslated regions of pIgR mRNA (open bars). B. Nucleotide sequence derived from cDNA clones hpIgR-1 and hpIgR-2. Numbering is relative to the start site of translation (position +1); negative numbers are assigned to the 5'-untranslated region. The amino acids are given below the nucleotide sequence in three-letter code. The N-terminal lys residue of the mature protein (amino acid 19 of the primary translation product) is underlined.
and rabbit (39) pIgR is shown in Fig. 3.3. Identity with the mouse sequence is indicated by dashes. The leader peptide, domains (one-six) that make up the extracellular region of pIgR, and the transmembrane and the cytoplasmic regions of the molecule are indicated by lines and arrows. The numbering system identifies the individual amino acids of the mouse pIgR and includes the leader peptide sequence. A summary of this alignment is shown in Table 3.1.

A comparison of the predicted amino acid sequences of mouse and rabbit pIgR showed them to be the least identical (Table 3.1). In contrast, mouse and rat pIgR share a high degree of homology. The transmembrane and cytoplasmic regions of mouse and rat pIgR are 96% and 92% identical, respectively, while the extracellular regions are only 85% identical. The mouse and human pIgR transmembrane, cytoplasmic and extracellular regions are 83%, 72% and only 64% identical, respectively. These regions in the mouse and rabbit are 78%, 68% and only 48% identical. Although inter-species identity appears to be lowest in the extracellular or secretory component region of the molecule, many of the amino acid substitutions are conservative changes, making SC homology more striking when the overall conservation of the amino acid sequence of the polypeptide is considered (mouse vs. rat, 92% conserved; mouse vs human, 82% conserved; mouse vs. rabbit, 68% conserved). Homology within the extracellular region is strikingly clustered in domain one which
Fig. 3.3. Interspecies alignment of polymeric immunoglobulin receptor amino acid sequences. An alignment of mouse (Fig. 3.1), rat (40), human (Fig. 3.2) and rabbit (39) pIgR sequences was performed using the AALIGN program. The numbering system identifies the individual amino acids of mouse pIgR and includes the leader peptide sequence. The leader peptide, extracellular immunoglobulin-like domains, transmembrane and cytoplasmic regions of the molecule are indicated by lines and arrows. Dashes indicate identity with the mouse pIgR sequence. The putative consensus noncovalent binding site of polymeric Ig to pIgR in domain one is boxed (46). Boxed residues in the large cytoplasmic region of pIgR denote conserved signals required for transepithelial trafficking (basolateral targeting/transcytosis/internalization) of pIgR.
Table 3.1. Amino acid homology of the polymeric immunoglobulin receptor is greatest in regions of the molecule involved in pIg binding and transcytosis

<table>
<thead>
<tr>
<th>Extracellular Region$^1$</th>
<th>Domain One</th>
<th>Domains Two-Five</th>
<th>Domain Six</th>
<th>All Domains</th>
<th>Transmembrane Region</th>
<th>Cytoplasmic$^2$ Region</th>
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<tbody>
<tr>
<td><strong>Mouse vs.</strong></td>
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<tr>
<td>Rat:</td>
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<tr>
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<tr>
<td>%Identical</td>
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<td>47</td>
<td>64</td>
<td>83</td>
<td>72</td>
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<tr>
<td>%Conserved</td>
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<td>81</td>
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<tr>
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<tr>
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<td>46</td>
<td>35</td>
<td>48</td>
<td>78</td>
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<td>66</td>
<td>59</td>
<td>68</td>
<td>91</td>
<td>88</td>
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</table>

$^1$Extracellular domains one-five have homology to immunoglobulin (Ig) variable domains. Domain one was analyzed independently because it is believed to contain the IgA-binding site. Domain six represents the connecting peptide between the Ig homology units and transmembrane region.

$^2$The cytoplasmic region contains signals for intracellular trafficking.
contains the putative consensus noncovalent binding site of pIg (see boxed domain one residues in Fig. 3.3) (46).

Other groupings of pIgR inter-species homology are apparent in the large cytoplasmic region which has been shown to contain signals important in receptor trafficking (boxed residues, Fig. 3.3). There is one conserved cluster which lies near the transmembrane region of pIgR. This 14 amino acid segment was shown to be responsible for basolateral sorting of pIgR (49). Several highly conserved serine residues and a conserved tyrosine residue are present in this region. One conserved serine residue in this segment (Ser$^{664}$ of the rabbit pIgR, corresponding to Ser$^{680}$ of mouse pIgR) has been shown to be required for transcytosis of pIgR only in the absence of dIgA (52). The conserved tyrosine residue (Tyr$^{668}$ of the rabbit pIgR, corresponding to Tyr$^{684}$ of mouse pIgR) has been shown to function as a pIgR internalization signal (113). A second clustering of inter-species homology lies at the pIgR C-terminus. This region has been shown to function in receptor endocytosis (50).

**Mouse pIgR mRNA is expressed in liver and intestinal epithelial cells but not in spleen**

To examine the tissue specificity of murine pIgR mRNA expression, a Northern blot analysis using a probe corresponding to nucleotides 431-770 (see Fig. 3.1) of the mouse pIgR coding sequence was performed. A single mouse pIgR transcript of
approximately 3.9 kb (Fig. 3.4) was detected in total cellular RNA prepared from isolated intestinal epithelial cells and liver. This finding suggests that the murine pIgR mRNA is not differentially processed. The mRNA for pIgR appears to be more abundant in mouse liver than in intestinal epithelial cells. Conversely, pIgR mRNA was not detected in total cellular RNA isolated from mouse spleen.

Discussion

Both mouse and human cDNAs containing the complete coding sequence of pIgR have been isolated. The availability of pIgR cDNAs from four different species allows identification of areas within the receptor that have been preferentially conserved. PIG has been shown to bind to the extracellular region of the receptor which is composed of five immunoglobulin-like domains and a sixth more distantly related domain (39). In this thesis study, domain one was observed to be the most highly conserved of all the immunoglobulin-like domains of the receptor (Table 3.1). Domain one has been shown to be responsible for high affinity noncovalent binding of dimeric IgA to rabbit pIgR (45). Using tryptic human SC fragments and synthetic peptides, Bakos et al. have localized the pIg-binding site of human pIgR to a specific 23 residue segment of domain one (46). This region (boxed, in Fig. 3.3) is strikingly similar in all four species examined. Synthetic peptides containing the 23 amino acid pIgR-binding site
Fig. 3.4. Polymeric immunoglobulin receptor mRNA is expressed in mouse liver and intestinal epithelial cells. Total cellular RNA (normalized to give equivalent actin signals) was hybridized with $^{32}$P-labeled cDNA probes for mouse pIgR and actin. Analysis of RNA from murine intestinal epithelial cells (EC) and liver (L) demonstrates expression of a 3.9 kb pIgR mRNA. Analysis of RNA from murine spleen (S) demonstrates the absence of pIgR mRNA expression.
apparently bind pIg. However, tryptic fragments containing the first extracellular domain of the receptor bind pIg with higher affinity (46). This finding may reflect the importance of the disulfide-stabilized conformation of the pIg-binding site. A cysteine residue (Cys$^{40}$ of the mouse pIgR) located within the pIg-binding site participates in the formation of a disulfide bond with another highly conserved cysteine residue of domain one (Cys$^{110}$) to form the Ig-like structure of this domain (114).

Several highly conserved residues are located adjacent to the pIg-binding site (see Fig. 3.3). Beale and Coadwell have used computerized predictions of secondary structure and three dimensional model building to predict the tertiary structures of the extracellular domains of human and rabbit pIgR (115). Their models for human and rabbit pIgR domain one suggest that the conserved cysteine (Cys$^{56}$ of mouse pIgR) and arginine residues that lie adjacent to the pIg-binding site may be involved in maintenance of the structure of this region. Cys$^{56}$ has been shown to be involved in a disulfide bond with Cys$^{64}$ (conserved in all four species examined), contributing to the structure of pIgR domain one (114).

The transmembrane portion of pIgR exhibits a high degree of interspecies conservation (>87%, Table 3.1). This may reflect the necessary inclusion of hydrophobic residues in this membrane-spanning domain. Mostov et al. have noticed a proline residue in this region of the rabbit receptor and suggested that
the pIgR molecule may "kink" as it crosses the membrane (39). This proline residue is conserved in all four species that were compared.

Although the sorting signals of pIgR appear to be confined to the cytoplasmic tail of the receptor, it has recently been demonstrated that interaction of pIg with the extracellular portion of the receptor may affect receptor trafficking. In the absence of dIgA, pIgR transcytosis was shown to be dependent upon phosphorylation of a serine residue (Ser^{664} of the rabbit pIgR, corresponding to Ser^{680} of mouse pIgR, Fig. 3.3) located in the cytoplasmic tail of the receptor (52). One speculation is that pIg binding to the extracellular portion of the receptor may induce a conformational change in the receptor, possibly involving the membrane-spanning region of the receptor. In support of this speculation, the portion of the cytoplasmic tail of pIgR, lying adjacent to the transmembrane domain and containing the serine residue implicated in entry of uncomplexed pIgR into the transcytotic pathway, was observed to be very similar in all four species. Another explanation for the interspecies similarity seen in this portion of the receptor tail is that this region has been shown to contain a 14-amino acid basolateral sorting signal (boxed, in Fig. 3.3) (49).

In general, the cytoplasmic region of pIgR which contains important signals for intracellular trafficking is well conserved across species (>88%, Table 3.1). All four pIgR species in this
study show a long stretch of 36 amino acids located at the C-terminus of the cytoplasmic tail which are nearly identical. Deletion of 30 of these C-terminal residues (boxed in Fig. 3.3) has been shown to reduce the rate of internalization of the receptor from the basolateral cell surface (50). A conserved tyrosine residue (Tyr$^{734}$ of the rabbit pIgR, corresponding to Tyr$^{750}$ of mouse pIgR) located in this region has been shown to play an important role in receptor endocytosis (113). This region also contains a conserved serine residue (Ser$^{726}$ of the rabbit pIgR, corresponding to Ser$^{742}$ of mouse pIgR) that has been shown to function in the transcytosis of pIgR (52). The function of the remaining six amino acids, identical in all four pIgR species, is unknown. These residues lie very close to Ser$^{726/742}$ and four out of six of these amino acids are basic. This charge clustering may contribute in some way to the phosphorylation event which occurs at this site.

By Northern blot analysis, the presence of a single murine pIgR transcript of approximately 3.9 kb was demonstrated. The rabbit pIgR has been shown by biochemical and Northern blot analysis to exist in both high- and low-molecular weight forms (47). The high-molecular weight form includes all of the extracellular domains of pIgR while the low-molecular weight form, which arises by alternative splicing of the rabbit pIgR mRNA, does not contain the second and third extracellular domains. Both human (41) and rat (40) pIgR have been shown to
exist in only one form, analogous to the high-molecular weight form in rabbit. Given the high degree of homology between mouse and rat pIgR, it was possible that mouse pIgR might also exist in a single high-molecular weight form. The probe used in the Northern blot analysis shown in Fig. 3.4 included only sequences from domains two and three, and would only detect the high-molecular weight form of the receptor. To detect both forms of the receptor, the analysis was repeated using a probe containing domain one sequences. Again, only the high-molecular weight pIgR form was observed (data not shown), suggesting that the mouse pIgR mRNA is not alternatively spliced.

Although pIgR mRNA was detected in murine liver and intestinal epithelial cells, it was not detected in spleen. The absence of pIgR mRNA expression has also been observed for human spleen (41). As FcαR mRNAs would be expressed by the neutrophils, macrophages and lymphocytes present in spleen tissue, the absence of pIgR mRNA expression in spleen demonstrates that the pIgR mRNA is distinct from mRNAs encoding Fc receptors for IgA (18).

In conclusion, mouse and human cDNAs containing 5'- and 3'-untranslated, and full length coding sequences of pIgR have been isolated. The predicted amino acid sequences of these cDNAs were aligned with those of rat and rabbit, and a high degree of amino acid sequence conservation was observed in regions of the pIgR molecule that are known to be functionally important. These
regions include the pIg-binding site in extracellular domain one and intracellular trafficking signals in the cytoplasmic region of pIgR. The tissue specificity of murine pIgR expression was also examined. pIgR mRNA is expressed in murine liver and intestinal epithelial cells, but not in spleen. These experiments confirm earlier immunohistochemical data on the tissue distribution of mouse pIgR (55). Very little is currently known concerning the mechanisms governing patterns of pIgR expression. As this receptor does not recycle, the mucosal sIg response may be regulated at least in part by the regulation of pIgR production. Isolation of the cDNAs, reported in this chapter, has provided probes for investigation of mechanisms of pIgR expression, including isolation of the promoter proximal region of the human pIgR gene (Chapter 5). Availability of the murine receptor will allow construction of transgenic and genetically deficient mice to more closely examine pIgR function and regulation.
CHAPTER 4

INTERFERON-γ INDUCES POLYMERIC IMMUNOGLOBULIN RECEPTOR mRNA IN HUMAN INTESTINAL EPITHELIAL CELLS BY A PROTEIN SYNTHESIS DEPENDENT MECHANISM
Abstract

Transport of secretory IgA into external fluids is mediated by the polymeric immunoglobulin receptor on the surface of mucosal epithelial cells. This study examines the mechanism by which interferon-γ induces pIgR expression in HT-29.74 cells, a subclone of the HT-29 cell-line selected for high concentrations of pIgR. A sensitive ribonuclease protection assay for pIgR mRNA was developed and used to determine if induction of pIgR by IFN-γ is mediated by accumulation of pIgR mRNA. pIgR mRNA increased 7-fold in response to IFN-γ, reaching a plateau at 24 h. Concentrations of pIgR protein also increased, but the major increase was not observed until 48 h following stimulation with IFN-γ. Cycloheximide treatment abolished the IFN-γ induced increase in pIgR mRNA, indicating that induction of pIgR mRNA by IFN-γ requires de novo protein synthesis. These results suggest that induction of pIgR expression by IFN-γ involves an increase in steady-state concentrations of pIgR mRNA via a protein synthesis dependent mechanism.

Introduction

Secretory IgA is the first line of immune defense, interacting with a variety of inhaled and ingested antigens (8, 9, 84, 116). Derived primarily from plasma cells in the lamina propria of mucous membranes, the daily production of IgA exceeds that of
all other Ig isotypes (9, 84, 116). Transport of secretory IgA into external fluids is mediated by the polymeric immunoglobulin receptor, also known as secretory component, on mucosal epithelial cells (12, 62, 117, 118). In rodents, pIgR on hepatocytes mediates transcytosis of polymeric IgA from blood to bile (32, 34, 86-88). Our laboratory has recently demonstrated that pIgR also mediates epithelial transcytosis of IgA immune complexes (22).

Therefore, mucosal IgA may serve a dual role of acting as an immunological barrier, combining with antigens external to body tissues (8, 9, 84, 116), as well as mediating the direct elimination of antigens at sites where IgA immune complexes are likely to form.

An increase in the production of pIgR at sites of infection or inflammation should enhance local rates of transcytosis of both IgA and IgA immune complexes. In the human colon adenocarcinoma cell-line HT-29, the expression of pIgR has been shown to increase in response to IFN-γ (65, 67, 68), tumor necrosis factor-α (66, 67) and interleukin-4 (68). Furthermore, direct application of IFN-γ to the uterine lumen in rats resulted in increased production of pIgR by uterine epithelial cells in vivo (119). It has been suggested that release of cytokines by locally activated mononuclear cells leads to increased expression of pIgR by mucosal epithelial cells, thus promoting transcytosis of IgA and IgA immune complexes and enhancing the availability of IgA for local defense functions. However, the molecular mechanisms of
induction of pIgR by cytokines remain to be elucidated. Using a sensitive ribonuclease protection assay, it has now been demonstrated that induction of pIgR expression by IFN-\(\gamma\) in the HT-29.74 cell-line involves an increase in steady-state concentrations of pIgR mRNA. Cycloheximide treatment abolished the IFN-\(\gamma\) induced increase in pIgR mRNA, suggesting that induction of pIgR mRNA by IFN-\(\gamma\) requires \textit{de novo} protein synthesis.

Materials and Methods

Cell culture

The derivation of the HT-29.74 cell-line, which was selected for high pIgR expression, has previously been described (64). HT-29.74 cells were induced to differentiate to an enterocytic phenotype by culturing in Leibovitz's L-15 medium (glucose-free) containing 5 mM galactose, 6 mM sodium pyruvate, 1 mM L-glutamine, 24 mM NaHCO\(_3\), 20 mM HEPES, pH 7.2, 100 U/ml penicillin, 100 \(\mu\)g/ml streptomycin, 250 ng/ml amphotericin B, and 10\% dialyzed FCS (64). All reagents for cell culture were obtained from GibcoBRL. Cells were plated at a density of 1.5 x 10\(^5\)/cm\(^2\) at the initiation of each experiment. As indicated in the figure legends, cells were treated with recombinant human IFN-\(\gamma\) (>98\% pure, 1 x 10\(^7\) U/mg protein; AmGen Biologicals, Thousand Oaks, CA), diluted in culture medium.
Measurement of cell-associated pIgR and released SC

Concentrations of pIgR in cell lysates and SC in culture supernatants were determined by ELISA as described in chapter two (64). Briefly, microtiter plates were coated with guinea pig antiserum to human SC, then incubated either with human SC (purified from colostrum) as standard or with experimental samples. Purified SC was diluted in culture medium for assay of culture supernatants or in cell lysis buffer (0.1 M sodium phosphate, pH 7.5, 1% (v/v) Tween-80, 0.5 M phenyl methyl sulfonyl fluoride, 5 mM EDTA and 25 kallikrein-inhibitor units of aprotinin/ml). Bound SC was detected by incubating with rabbit antiserum to human SC, followed by alkaline phosphatase-conjugated goat antirabbit IgG (Boehringer Mannheim Corp.) and phosphatase substrate (Sigma). Cell counts were obtained when cell lysates or culture supernatants were harvested, and data were expressed as ng pIgR or SC/10^5 cells.

Measurement of pIgR mRNA

A ribonuclease protection assay was developed as a sensitive and quantitative method for measuring steady-state concentrations of pIgR mRNA. A 183-bp cDNA fragment spanning nucleotides 214-396 of the human pIgR coding sequence (see Fig. 3.2) was generated by PCR (GeneAmp® PCR reagent kit, Perkin Elmer Cetus). The final concentrations of the reactants in the 100 μl reaction mixture were as follows: 10 mM Tris-HCl (pH 8.3); 50
mM KCl; 1.5 mM MgCl₂; 0.001% (w/v) gelatin; 200 μM each dATP, dGTP, dTTP and dCTP; 1 μM each primer; 2.5 U of Taq polymerase and 20 ng of template cDNA/ 100 μl. The amplification cycle used for the PCR is described in chapter three. Template cDNA for the PCR was prepared by reverse transcription of poly A⁺ RNA isolated from HT-29.74 cells, using Moloney murine leukemia virus reverse transcriptase (GibcoBRL) and oligo dT as primer. The synthetic oligonucleotides used as primers corresponded to nucleotides 214-238 and the reverse complement of residues 372-396 of the human pIgR coding sequence. The primers contained 9 nt extensions at their 5' ends which generated Kpn I and BamH I sites at the 5' and 3' ends, respectively, of the amplified product. The amplified cDNA was purified by polyacrylamide gel electrophoresis and inserted into the Kpn I and BamH I sites of pBluescript (Stratagene). The pIgR-pBluescript plasmid was linearized with Kpn I, and a 239 nt ³²P-labeled antisense RNA probe was synthesized at 37°C for 30 min. using 1 μg linearized plasmid, 10 U T3 polymerase, 10 mM each of ATP, CTP and GTP and 50 μCi of [³²P]-UTP (800 Ci/mmol; NEN Research Products) in a final volume of 25 μl (RNA Transcription Kit, Stratagene). The labeled probe (average specific activity, 4 x 10⁷ cpm/pmol) was separated from unincorporated nucleotides by electrophoresis on a 6% polyacrylamide gel containing 7 M urea (Gelmix-6, GibcoBRL), eluted with a solution of 2 M
ammonium acetate, 1% SDS and 25 μg/ml yeast transfer RNA, and ethanol precipitated.

Total cellular RNA was extracted from cultured cells with guanidinium isothiocyanate and purified by centrifugation on cesium chloride gradients (120). The RNase protection assay was performed essentially as described (121, 122). Varying amounts of total cellular RNA (0.5 - 5 μg) were precipitated with ethanol and air-dried. RNA precipitates were dissolved in 30 μl hybridization buffer (40 mM 1,4-piperazine diethanesulfonic acid (PIPS), pH 6.4, 400 mM NaCl, 1 mM EDTA, 80% (v/v) formamide) containing 5 x 10⁵ cpm of the ³²P-labeled pIgR antisense RNA probe. The hybridization mixture was incubated at 85°C for 10 min. to denature the RNA, then allowed to anneal overnight at 45°C. To each hybridization mixture was added 300 μl of ribonuclease digestion mixture (10 mM Tris-HCl, pH 7.4, 300 mM NaCl, 5 mM EDTA, 2 μg/ml RNase T1 (US Biochemical) and 40 μg/ml RNase A (US Biochemical)). Samples were digested at 30°C for 60 min., then treated at 37°C for 30 min. with 20 μl of 10% (w/v) SDS and 10 μl of a 10 mg/ml solution of proteinase K (Boehringer Mannheim Corp.). RNA was then extracted with phenol/chloroform, ethanol-precipitated and air-dried. RNA precipitates were dissolved in 10 μl of 80% (v/v) formamide, 10 mM EDTA, separated by electrophoresis as described above, and visualized by autoradiography on Kodak XAR-5 film. The size of the protected fragment was determined by comparison to ³²P-
labeled DNA molecular size standards (ΦX174 RF DNA/Hae III fragments; GibcoBRL) and to undigested, 239 nt $^{32}$P-labeled RNA probe. The concentration of pIgR mRNA was quantified, as indicated in the figure legends, either by excision and scintillation counting of the 183 nt, pIgR-specific band or by densitometry of the autoradiogram.

**Treatment of HT-29.74 cells with cycloheximide to block protein synthesis**

To determine if induction of pIgR mRNA by IFN-γ requires protein synthesis, HT-29.74 cells were treated for 6 h with 2 mg/ml cycloheximide (Sigma). This concentration of cycloheximide was determined to block greater than 99% of protein synthesis by measuring incorporation of $[^{35}$S]$\text{-}$methionine (NEN Research Products) into TCA-precipitable material (data not shown). Cell viability after cycloheximide treatment, determined by trypan blue exclusion, averaged 98%.

**Statistical analyses**

In Fig. 4.3, the correlation between input RNA and detected amounts of pIgR mRNA was determined by linear regression analysis. In Fig. 4.5, main effects of IFN-γ and cycloheximide were tested by analysis of variance, and differences among individual groups were tested by the method of least significant difference.
All analyses were performed using the Complete Statistical System for IBM PCs (Statsoft, Tulsa, OK).

Results

Expression of the polymeric immunoglobulin receptor by HT-29.74 cells is induced by IFN-γ

The isolation of a subclone of the HT-29 human colon carcinoma cell-line, HT-29.74, selected for high expression of pIgR has been previously reported by this laboratory (64). HT-29.74 cells were induced to differentiate to an enterocytic phenotype by growth in glucose-free medium, resulting in increased expression of pIgR (64). Treatment of HT-29.74 cells with IFN-γ resulted in a dramatic, dose-dependent increase in release of SC (the cleaved ectoplasmonic domain of pIgR), peaking at 100 U/ml (Fig. 4.1). Similar increases in cell-associated pIgR were observed (data not shown), indicating that increased SC concentrations in conditioned media from IFN-γ treated cells resulted from increases in total cellular content of pIgR rather than enhanced release of SC. The major increase in expression of pIgR was not seen until 2 days following stimulation with IFN-γ, suggesting that there may be one or more critical rate-limiting steps leading to induction of pIgR expression (see below).
Figure 4.1. Dose-dependent induction of pIgR expression by IFN-γ. HT-29.74 cells were cultured for 4 days in the presence or absence of varying doses of recombinant human IFN-γ. Media were changed daily, and released SC was measured in culture supernatants by ELISA. Bars represent cultures treated with (left to right) 0, 10, 31.6, 100, 316 and 1000 U/ml IFN-γ, respectively. Data are expressed as mean ± S.D. (n = 4).
Induction of pIgR by IFN-γ is associated with increased steady-state concentrations of pIgR mRNA

To investigate the mechanism by which IFN-γ induces the expression of pIgR, a sensitive ribonuclease protection assay for detection of pIgR mRNA was developed. Total cellular RNA was extracted from HT-29.74 cells cultured for 2 days in the presence or absence of 100 U/ml IFN-γ, hybridized with a 239 nt 32P-labeled antisense RNA probe, and digested with ribonucleases A and T1. The band at 183 nt represents fragments of the probe protected by hybridization to pIgR mRNA (Fig. 4.2). This assay was sufficiently sensitive to detect pIgR mRNA in 5 μg of total cellular RNA from unstimulated HT-29.74 cells. Stimulation of HT-29.74 cells for 48 h with IFN-γ resulted in significant accumulation of pIgR mRNA (Fig. 4.2). To establish the validity of the ribonuclease protection assay for the quantification of pIgR mRNA, varying concentrations of total cellular RNA from IFN-γ stimulated HT-29.74 cells were analyzed (Fig. 4.3). The detected amount of pIgR mRNA was linear over the range of 0.1 - 5 μg of total cellular RNA (r = .999), demonstrating a high degree of precision of the assay.

To determine if the increase in pIgR mRNA could account for the induction of pIgR expression by IFN-γ, concentrations of pIgR mRNA and protein were quantified in HT-29.74 cells cultured for varying times in the presence or absence of 100 U/ml IFN-γ (Fig. 4.4). While a modest increase in pIgR mRNA was observed during
Figure 4.2. Ribonuclease protection assay for detection of pIgR mRNA. Total cellular RNA was extracted from HT-29.74 cells cultured for 2 days in the presence or absence of 100 U/ml IFN-γ. Five μg of total cellular RNA was hybridized with a 239 nt \textsuperscript{32}P-labeled antisense RNA probe, then treated with ribonucleases A and T1 to remove unhybridized probe. After digestion, RNA was denatured in formamide and analyzed by electrophoresis on a 6% polyacrylamide gel containing 7 M urea. Sizes of the bands were determined by comparison to \textsuperscript{32}P-labeled molecular size standards (not shown). The band at 183 nt represents fragments of the probe protected by hybridization to pIgR mRNA. No protection was seen when the probe was incubated with yeast transfer RNA (tRNA).
Figure 4.3. Quantification of pIgR mRNA by ribonuclease protection assay. Varying amounts of total cellular RNA from HT-29.74 cells treated for 2 days with 100 U/ml IFN-γ were analyzed by ribonuclease protection as described in the legend to Fig. 4.2. The amounts of the 183 nt protected fragments were determined by scintillation counting of the excised bands. Correlation between the amount of input RNA and detected amounts of pIgR mRNA was determined by linear regression analysis.
\[ r = 0.999 \]
the initial 12 h, the greatest increase was observed between 12 and 24 h, at which time concentrations of pIgR mRNA were 7-fold greater in IFN-γ stimulated than in unstimulated cells. Stimulation with IFN-γ also caused a 7-fold increase in pIgR protein, but the major increase not seen until 48 h (see also Fig. 4.1). The observation that induction of pIgR mRNA preceded but corresponded in magnitude to that of pIgR protein suggests that the mechanism of induction of pIgR expression by IFN-γ involves increases in steady-state concentrations of pIgR mRNA.

**Induction of pIgR mRNA by IFN-γ requires *de novo* protein synthesis**

The major increase in pIgR mRNA requires 24 h following treatment with IFN-γ (Fig. 4.4). This observed increase in steady-state pIgR mRNA concentration might involve changes in pIgR mRNA half-life and/or synthesis. Either of these mechanisms of induction might require *de novo* protein synthesis. To test whether induction of pIgR mRNA requires *de novo* protein synthesis, HT-29.74 cells were treated with a concentration of cycloheximide that was determined to block greater than 99% of protein synthesis. Treatments were limited to 6 h intervals to minimize cytotoxic effects of cycloheximide; cell viability averaged 98% following cycloheximide treatment. Preliminary experiments indicated that the rate of protein synthesis had recovered significantly by 1 h following withdrawal of cycloheximide (data
Figure 4.4. Time course of induction of pIgR mRNA and protein by IFN-γ. HT-29.74 cells (two cultures/time point) were cultured for varying times in the presence (squares) or absence (circles) of 100 U/ml IFN-γ. Two μg of total cellular RNA were analyzed for pIgR mRNA (solid lines) as described in the legend to Fig. 4.2, and arbitrary units of pIgR mRNA were determined by densitometry. Total pIgR protein, analyzed by ELISA (dashed lines), is the sum of the amount of pIgR in cell lysates and the amount of SC in culture supernatants. Data are expressed as means of duplicate observations.
not shown). To determine the critical period for de novo protein synthesis, cells were treated with cycloheximide during hours 0-6, 3-9 or 6-12 of the experiment. Cells were stimulated with IFN-\(\gamma\) during hours 0-6 of the experiment; this pulse of IFN-\(\gamma\) was used instead of continuous stimulation so that cycloheximide treatment would either coincide with or follow IFN-\(\gamma\) stimulation. Media were changed every 3 h for all groups to ensure that neither the IFN-\(\gamma\) nor the cycloheximide were exhausted. Total cellular RNA was collected from all groups at 24 h after initiation of IFN-\(\gamma\) treatment, and concentrations of pIgR mRNA were quantified (Fig. 4.5). Blocking protein synthesis completely prevented the induction of pIgR mRNA by IFN-\(\gamma\), regardless of when during the first 12 h cycloheximide was given. These results indicate that the mechanism of induction of pIgR mRNA by IFN-\(\gamma\) requires de novo protein synthesis.

**Discussion**

In locations such as the respiratory and intestinal tracts, the presence of large numbers of mucosal epithelial cells expressing pIgR provides a high capacity for constitutive transport of IgA produced by local plasma cells. However, induction of pIgR expression by cytokines at focused sites of immune activation would lead to an increased targeting of IgA to sites where it can participate most effectively in the elimination of potentially noxious antigens. Characterizing the molecular mechanisms by
Figure 4.5. Induction of pIgR mRNA by IFN-γ is blocked by cycloheximide. HT-29.74 cells were cultured during hours 0-6 h of the experiment in the presence (hatched bars) or absence (solid bars) of 100 U/ml IFN-γ, then cultured for an additional 18 h in medium without IFN-γ. Individual groups were treated with 2 mg/ml cycloheximide during h 0-6, 3-9 or 6-12 of the experiment; all cultures were harvested at 24 h. Five μg of total cellular RNA from each culture were analyzed for pIgR mRNA as described in the legend to Fig. 4.2, and arbitrary units of pIgR mRNA were determined by densitometry. Data are expressed as mean ± S.D. (n = 4). A significant increase in pIgR mRNA in response to IFN-γ was observed in the group not treated with cycloheximide (p < .0001); no significant effect of IFN-γ was observed among any groups treated with cycloheximide.
which cytokines such as IFN-γ induce pIgR expression will increase our understanding of ways in which communication between activated immunocytes and epithelial cells enhances mucosal immune responses.

HT-29 cells and their derived subclones are the only established human cell-lines that have been shown to express pIgR constitutively (12, 62, 64, 102, 117). However, both the percentage of positive cells and the basal levels of expression of pIgR are low (64, 65), requiring sensitive methods for detection of pIgR mRNA and protein. While Northern blot analysis has been used to detect pIgR mRNA in human tissues (41), it was not possible to detect pIgR mRNA in unstimulated HT-29.74 cells using this technique (data not shown). Therefore, a ribonuclease protection assay with sufficient sensitivity to quantify pIgR mRNA in as little as 5 μg of total cellular RNA from unstimulated HT-29.74 cells was developed (Fig. 4.2).

This study demonstrates that induction of pIgR expression by IFN-γ involves an increase in pIgR mRNA via a protein synthesis dependent mechanism. The increase in pIgR mRNA required 12 h following initiation of IFN-γ treatment (Fig. 4.4); furthermore, blocking protein synthesis at any time during this 12 h period abrogated the induction by IFN-γ (Fig. 4.5). These observations suggest that there may be a cascade of events leading to the accumulation of pIgR mRNA, one or more of which requires *de novo* protein synthesis. The newly synthesized
protein(s) could be transcription factor(s) that interact with IFN-γ response elements in the pIgR gene or, alternatively, factor(s) that increase the stability of pIgR mRNA. A delay of 24 h was observed between the peak in pIgR mRNA and the increase in expression of pIgR protein (Figs. 4.1 and 4.4). This time course suggests that additional mechanisms, perhaps at the level of translation, may be operative in the induction of pIgR expression by IFN-γ. The sequence of human pIgR cDNA indicates that the 5'- and 3'-untranslated regions of pIgR mRNA are unusually long, at least 180 and 443 bp, respectively (Fig. 3.2), raising the possibility that elements may be present within these noncoding regions that regulate either the stability or rate of translation of pIgR mRNA.

A number of similarities are apparent between the regulation of expression of pIgR and MHC class II antigens. Both are induced by IFN-γ and tumor necrosis factor-α in HT-29 cells, with nearly identical kinetics (67; and data not shown). It has been suggested that induction of MHC class II genes in intestinal epithelial cells by cytokines may facilitate the role of these cells in presentation of mucosal antigens to intraepithelial T cells (26). Coordinate regulation of pIgR and MHC class II genes could simultaneously enhance the participation of epithelial cells in both the activation and effector phases of the mucosal immune response. Induction of MHC class II genes by IFN-γ in a variety of cell types has been shown to be mediated at least in part by increases in the rates of transcription of these genes (83, 123,
Accumulation of MHC class II mRNA following stimulation with IFN-γ requires de novo protein synthesis in many cell types; whether these newly synthesized proteins exert their effects at the transcriptional or post-transcriptional level is controversial (83, 123, 124). IFN-γ response elements have been mapped to the proximal 5'-flanking region of human and murine MHC class II genes, and regulatory proteins binding within these regions have been identified (reviewed in (82)). Mapping the putative promoter-regulatory region of the human pIgR gene (Chapter 5) has identified similar elements, consistent with linked regulation of the two sets of genes. However, expression of pIgR is restricted to mucosal epithelial cells (5), suggesting that there is also a unique, cell-type specific mechanism for control of pIgR expression. Identification of the mechanism for maintenance of cell-type specific induction of pIgR by IFN-γ may reveal a novel pathway for regulation of gene expression.
CHAPTER 5

MOLECULAR CLONING OF THE PROMOTER
PROXIMAL REGION OF THE HUMAN
POLYMERIC IMMUNOGLOBULIN RECEPTOR GENE
Abstract

The polymeric immunoglobulin receptor mediates the transcytosis of dimeric IgA and pentameric IgM into external secretions. Expression of the receptor is tissue specific and inducible by cytokines such as IFN-γ, IL-4 and TNF-α, but little is known concerning the molecular mechanisms involved. To identify DNA sequence elements that may be involved in control of pIgR expression, three overlapping genomic clones containing the 5'-flanking region of the human pIgR gene were isolated. By primer extension and ribonuclease protection assays using RNA extracted from human intestinal epithelial cells, the transcriptional start site of the gene was identified. Analysis of the sequence of the promoter proximal region of the human pIgR gene revealed potential cis-acting regulatory elements similar to the X and Y boxes of the MHC class II genes, and the absence of a classic TATA box.

Introduction

Secretory IgA operates in a variety of ways to provide immune defense at mucosal sites (16-18, 125). It acts as an immune barrier preventing entry of foreign antigens, neutralizes viruses, and binds immune complexes, carrying them across the epithelium to be excreted. Polymeric immunoglobulin receptor, also known as secretory component, is the transmembrane
glycoprotein that mediates the transport of polymeric IgA and IgM into secretions (11, 12). PIgR has been called "the sacrificial receptor" (36). Instead of recycling, it is cleaved during transcytosis and becomes an important part of the sIg molecule, possibly providing protection from degradation in the mucosal environment (13-15). Therefore, regulation of PIgR production may regulate, at least in part, the mucosal immune response.

The PIgR is expressed by a variety of human exocrine tissues including the gastrointestinal mucosa, salivary, lachrymal, and mammary glands (125). The major difference between PIgR expression in humans vs. rodents appears to be its tissue specificity. In rodents, PIgR is expressed in hepatocytes, in addition to the mucosal epithelia, and sIgA reaches the intestinal lumen via bile (32, 87, 88). The mechanisms that control the tissue specificity of PIgR expression have not been investigated.

Cytokines, acting locally, may enhance the mucosal IgA response by increasing PIgR production. Epithelial expression of PIgR protein has been shown to increase in response to IFN-γ, TNF-α, and IL-4 (65-68, 74). The induction of PIgR expression by IFN-γ has been shown to involve increases in PIgR mRNA (43, 70; see also Chapter 4). These increases were abolished by cycloheximide which suggests that de novo protein synthesis may be required to provide transcription factors that interact with IFN-γ response elements in the PIgR gene. Genomic DNAs encoding human PIgR have been cloned and a detailed analysis of
the intron-exon structure of the gene has been reported (43, 53, 54). However, the promoter region of the pIgR gene and the molecular mechanisms that control the induction of pIgR expression by cytokines have not been studied.

To begin to identify the DNA sequence elements involved in control of pIgR expression, genomic DNAs containing the 5'-flanking region of the human pIgR gene were isolated. Using primer extension and RNase protection assays, the transcriptional start site of the gene has been mapped. An analysis of the sequence of the promoter proximal region has identified several potential regulatory elements and revealed the absence of a classic TATA box.

Materials and Methods

Isolation of human pIgR genomic clones

To isolate genomic clones which might contain the 5'-flanking region of the human pIgR gene, approximately one million recombinants from a commercially prepared λFIX II human fibroblast genomic library (Catalog #946204, Stratagene) were screened as described in chapter three but using the bacterial host strain, SRB(P2). The probe that was used in this screening spanned 99 nucleotides (-161 to -63, Fig. 3.2; designated as nt +25 to +123 of exon one in Fig. 5.2B) of the 5'-UTR sequences of the human pIgR cDNA (43). This probe was simultaneously amplified and radiolabeled as described in chapter
three using the human pIgR cDNA, hpIgR-1 (see Fig. 3.2) as template DNA. The 5'-sense primer used in the PCR had the sequence 5' CAG TGC CCT GCC AGT AGC TCC TAG A 3', and the 3'-antisense primer had the sequence 5' CTT CCT TCT CTC CCG TTG ATC TGA CTT CAG 3'. Three human pIgR genomic DNA clones were identified.

**Southern blot analysis, and DNA sequencing**

λDNA was prepared from the plaque purified genomic clones by PEG precipitation (111) and digested with Not I in combination with either EcoR I, or BamH I, or both (GibcoBRL). After electrophoresis in 1% agarose, the DNA was depurinated in 2.5 N HCl, denatured in 1.5 M NaCl, 0.5 N NaOH, and transferred to Genescreen® (NEN Research Products) by capillary blotting. The filter was baked at 90°C for 2 hours, and prehybridized for 2 h at 42°C in 6X SSC, 5X Denhart's solution, 1% SDS and 100 μg/ml boiled salmon sperm DNA. Hybridization was performed at 42°C for 18 h in the same solution as the prehybridization with the addition of a 32P-end-labeled oligonucleotide, corresponding to nucleotides -161 to -137 of the 5'-UTR sequences of human pIgR cDNA (43; see also Fig. 3.2; or Fig. 5.2B, where this sequence has the numerical designation +25 to +49). The oligonucleotide was labeled using T4 polynucleotide kinase (GibcoBRL) and [32P]-ATP (3000 Ci/mmol; NEN Research Products) (122). The blot was washed in 6X SSPE, 0.1% SDS at 42°C and visualized by
autoradiography using an intensifying screen and Kodak XAR-5 film. Southern blot analysis revealed a 6 kb *EcoR I/BamHI* hybridizing fragment. This fragment was subcloned into pBluescript II (Stratagene) to create the plasmid, pIgR-pBS SK. Approximately 700 bp located at the 3' end of this fragment were sequenced by the dideoxy chain-termination method (109) using the Sequenase version 2.0 sequencing kit (United States Biochemical Corporation) (110). The primers used for sequencing included the primers used in the PCR and also primers specific for pBluescript vector sequences (described in chapter three). Sequence analyses, and homology searches against the GenBank DNA sequence database were performed using the ALIGN (DNASTAR, Inc.), MacVector (IBI, New Haven, CT) and BLAST (126) programs.

**Isolation of RNA from human intestinal epithelial cells**

The isolation of human intestinal epithelial cells using dispase has been described (127). Briefly, intestinal mucosal strips were treated at room temperature for 30 min. in 1.5 mg/ml dithiothreitol (DTT) solution, then 5-30 gms of tissue were placed in a 50-ml centrifuge tube containing 15 ml of dispase (Boehringer Mannheim Corp.) dissolved in RPMI 1640 (GibcoBRL) at a concentration of 3 mg/ml. The tissue was incubated at 37°C for 30 min., vortexed every 5 min., moved to a second tube of dispase solution and incubated again. Recovered cells were
pooled, washed in RPMI 1640 and incubated once in dispase solution as above with 1 mg/ml of DNase added (Sigma). The suspension was passed over a nylon wool column, and epithelial cells were purified by density gradient centrifugation for 20 min. at 20°C and 17,000g using 40% (v/v) Percoll (Pharmacia). Epithelial cells were collected from the interface and washed twice in RPMI 1640.

Total cellular RNA was isolated from epithelial cells by a modification of the technique of Chomczynski and Sacchi (112). Briefly, epithelial cells were lysed in RNAzol™ (Cinna/Biotechx), 0.1 volume of chloroform was added, and the mixture was shaken, followed by incubation on ice for 15 min. After centrifugation at 4°C for 20 min. at 12,000g, the aqueous phase was collected and precipitated overnight at -20°C by the addition of an equal volume of isopropanol. RNA was pelleted by centrifugation at 4°C for 30 min. at 12,000g, and resuspended in 10 mM Tris-HCl (pH 8.0), 1 mM EDTA.

**Primer extension analysis**

Primer extension reactions were performed according to established protocols (122) using two antisense oligonucleotides corresponding to regions of the human pIgR 5'-UTR, designated primer 1 (5' GCT ACT GGC AGG GCA CTG GAC CCT G 3') and primer 2 (5' CTT CCT TCT CTC CCG TTG ATC TGA CTT CAG 3'). Primers, end-labeled as described above, were hybridized with 10 µg of
total cellular RNA from human intestinal epithelial cells by incubation at 80°C for 10 min. in a solution containing 80% formamide, 0.4 M NaCl, 40 mM PIPES, pH 6.4, 1 mM EDTA, pH 8.0 followed by hybridization in the same solution for 18 h at 30°C. RNA:primer hybrids were coprecipitated and extended for 1 h at 42°C using SuperScript™ reverse transcriptase (GibcoBRL), a modified Moloney murine leukemia virus reverse transcriptase. The reaction was stopped and the RNA degraded by addition of EDTA (pH 8.0) and RNase A (Boehringer Mannheim Corp.), which had been boiled for 10 min. to inactivate DNase activity, to final concentrations of 20 mM and 40 μg/ml respectively, and incubating for 30 min. at 37°C. The primer extension products were precipitated, heated in denaturing buffer and size-fractionated by electrophoresis on a 6% polyacrylamide sequencing gel containing 7 M urea (Gelmix-6, GibcoBRL). Lengths of the extended products were determined by comparison to DNA sequencing reactions which were performed using the same oligonucleotide primers, and electrophoresed adjacent to the primer extension products.

Nuclease protection assay

The RNase protection assay was performed essentially as described in chapter four (43). The pIgR-pBS SK plasmid described above was linearized with Hind II (see Fig. 5.2B). A 610 nt 32P-labeled antisense RNA probe was synthesized at 37°C for
30 min. using 1 μg linearized plasmid, 10 U T3 polymerase, 10 mM each of ATP, CTP and GTP and 50 μCi of [\(^{32}\)P]-UTP (800 Ci/mmol; NEN Research Products) in a final volume of 25 μl (RNA Transcription Kit, Stratagene). The labeled probe was separated from unincorporated nucleotides by electrophoresis on a 6% polyacrylamide gel containing 7 M urea (Gelmix-6, GibcoBRL), eluted with a solution of 2 M ammonium acetate, 1% SDS and 25 μg/ml yeast transfer RNA, and ethanol precipitated.

Varying amounts of total cellular RNA (0.25 - 1.5 μg) from human intestinal epithelial cells were precipitated with ethanol and air-dried. RNA precipitates were dissolved in 30 μl hybridization buffer (40 mM PIPES, pH 6.4, 400 mM NaCl, 1 mM EDTA, 80% (v/v) formamide) containing 5 x 10^5 cpm of the \(^{32}\)P-labeled pIgR antisense RNA probe. The hybridization mixture was incubated at 85°C for 10 min. to denature the RNA, then allowed to anneal overnight at 45°C. To each hybridization mixture was added 300 μl of ribonuclease digestion mixture (10 mM Tris-HCl, pH 7.4, 300 mM NaCl, 5 mM EDTA, 2 μg/ml RNase T1 and 40 μg/ml RNase A (US Biochemical)). Samples were digested at 30°C for 60 min., then treated at 37°C for 30 min. with 20 μl of 10% (w/v) SDS and 10 μl of a 10 mg/ml solution of proteinase K (Boehringer Mannheim Corp.). RNA was then extracted with phenol/chloroform, ethanol-precipitated and air-dried. RNA precipitates were dissolved in 10 μl of 80% (v/v) formamide, 10 mM EDTA, separated by electrophoresis as described above, and
visualized by autoradiography on Kodak XAR-5 film. The size of the protected fragment was determined by comparison to $^{32}\text{P}$-labeled DNA molecular size standards (ΦX174 RF DNA/Hae III fragments, 100 and 123 bp ladders; GibcoBRL) and to the undigested 610 nt, $^{32}\text{P}$-labeled RNA probe. A DNA to RNA size conversion of 93% was used to estimate the size of the protected RNA fragment (128).

**Results**

**Identification of the start site of transcription of the human pIgR gene**

Primer extension analysis and RNase protection assay were used to determine the transcription initiation site of the human pIgR gene. The primer extension analysis was first performed using a primer complementary to sequences +25 to +49 (primer 1) (Fig. 5.2B). This end-labeled oligonucleotide was annealed to total cellular RNA isolated from human intestinal epithelial cells and then extended using reverse transcriptase. Electrophoresis revealed several primer extension products, including a short product that ran just above the unextended primer on the gel (data not shown). A second oligonucleotide, corresponding to sequences +94 to +123 (primer 2), was synthesized and the analysis was repeated. Primer extension using primer 2 yielded one prominent band (Fig. 5.1A). A sequencing reaction using primer 2 was performed on the pIgR-pBS SK plasmid (described
Figure 5.1. Primer extension and nuclease protection analyses to identify the start site of transcription of the human pIgR gene. A. Primer extension. A $^{32}$P-labeled antisense oligonucleotide (primer 2), corresponding to nucleotides +94 to +123, was hybridized to 10 $\mu$g of human intestinal epithelial cell RNA or yeast tRNA. The pIgR mRNA:primer DNA hybrid was extended using reverse transcriptase, and separated using denaturing PAGE. Analysis of RNA from human intestinal epithelial cells (HEC) demonstrates a single prominent band. A sequencing reaction using the same oligonucleotide primer is shown, allowing base specific identification of the primer-extended product. Nucleotides are as indicated (G, A, T, C). The corresponding sequence is given at the side. The deduced transcriptional start site of the pIgR gene is marked by an arrow. B. Nuclease protection assay. A 610 nt $^{32}$P-labeled pIgR antisense probe was prepared by *in vitro* transcription and hybridized at 45°C for approximately 16 hours with 1.5, 0.5 and 0.25 $\mu$g (Lanes 1, 2 and 3) of total cellular RNA from human intestinal epithelial cells (HEC) or yeast transfer RNA (tRNA). The RNA:RNA hybrids were digested with RNase and separated using 6% denaturing PAGE. The size of the protected fragment was determined by comparison to $^{32}$P-labeled molecular size standards (not shown). The band at 132 nt represents the probe fragment that was protected by hybridization to pIgR mRNA. No protection was seen when the probe was incubated with yeast tRNA alone.
in Materials and Methods) and run on the same gel to allow identification of a candidate nucleotide, "T", corresponding to the transcriptional start site for pIgR (see arrows in Figs. 5.1A and 5.2B). This site defines the length of the first exon of the pIgR gene as 132 nucleotides. Extended products were not observed for primer extension reactions where yeast tRNA was substituted for epithelial cell RNA (data not shown).

The results of the primer extension analysis were supported by an RNase protection assay using an in vitro transcribed pIgR RNA probe corresponding to the region between the Hinc II and BamH I sites which flank the deduced pIgR transcriptional start site. A single RNA fragment of approximately 132 nucleotides, as compared with RNA and DNA fragments of known sizes, was protected by hybridization to total RNA isolated from human intestinal epithelial cells (Fig. 5.1B). As a negative control, yeast tRNA alone did not protect a detectable fragment.

The 5'-flanking region of the human pIgR gene

To isolate a genomic DNA containing the 5'-flanking region of the human pIgR gene, a human fibroblast genomic library was screened with a probe which spanned 99 nucleotides (designated as nt +25 to +123 of exon one in Fig. 5.2B) of the 5'-UTR sequences of human pIgR (43). This screening led to the isolation of three pIgR genomic DNAs. By Southern blot analysis, a 6 kb EcoRI/BamHI fragment (Fig. 5.2A) which hybridized to an
Figure 5.2. The 5'-flanking region of the human pIgR gene. A. Physical map of a 6 kb DNA fragment containing the 5'-flanking region of the pIgR gene: (B) BamH I; (E) EcoR I. The position of exon one is indicated by an arrow. The location of the promoter proximal sequence is designated by a bar. B. The sequence of the promoter proximal region of the human pIgR gene. Numbering is relative to the deduced start site of transcription (position +1); negative numbers are assigned to the 5'-flanking region of the gene. The BamH I site used to subclone this fragment, the Hinc II site used to prepare the antisense RNA probe for the nuclease protection assay, and exon one which contains 5'-UTR sequences of the pIgR gene are underlined. Boxes indicate sequences similar to the S, X and Y box consensus motifs found in the promoter regions of MHC class II genes (82). Brackets indicate a sequence which is homologous to the promoter region of the bovine cytokeratin IV gene (129).
A

E

B

-550  TTCCCTGGAGCCTCTGGGACTGGTAAAGGGGCTTGCCAGGAGGTGTATAAG
-544  GGGCATTCTTGTCCAGGCTAAGTACACTAGGAAGGCTGGGAGGATTCAG
-494  AGCAACTGGGGATATGAGACCAAGGAAGACTCAGCACGCACCTCTGACCCT
-444  GTGCCCTCATCAGATGATGTCACCTCATTCAAATGACATTTGGCAGTTATTT
-394  TTAGAGCTAATACCGGGCTATATCCTCTCTACCTGTAGATTGTTATTACCA
-344  TCCCTCTTTTTCCAGATGAAAAGAAAATAGGAAGGTGACTTGGCCAAAGGTC
-294  TTGCCGCTAGAAAGCGACAGCAACAGCATCTTCCAGCTTGACATTCTGTCC
-244  CTCATCCGGAAGCGTCAACGATGGAGTTCCAAATGACAGAAGTGCTCCC
-194  "S-BOX"
-144  CAAGGTCGAAAGGAAACCCATGGGACGCGACGGCGAGATGGCCAAAATGGAAG
-94   "Y-BOX"
-44   "X-BOX"
+7   GAGTTTTCATTTTGGCACAGCCCTCGTCGCAGTGACTGCTCTAGA
+57   exin 1
+107  AACGGGAGAGAAAGGATGGCCTAAAATGTAAGTGTTACTGCTCTGGGATCC

intron BamHI
oligonucleotide containing human pIgR 5'-UTR sequences (nt +25 to +49 in Fig. 5.2B) was demonstrated in two of the genomic DNAs. This fragment was subcloned into pBluescript and a region consisting of 700 bp located at the 3' end of the fragment was sequenced. An exon containing the 5'-UTR sequences was found directly upstream of the BamH I restriction site (Fig. 5.2B). This finding is in agreement with the intron-exon map of the human pIgR gene reported by Krajci et al. (54).

Analysis of the sequence of the human pIgR 5'-flanking region (Fig. 5.2B), located with respect to the identified transcriptional start site, revealed several potential cis-acting enhancer elements. The typical TATA- and CAAT-box sequences, present in many eukaryotic promoters, were not found in the 5'-flanking region of the human pIgR gene. Sequences similar to the S, X and Y boxes, important regulatory elements of the promoter proximal regions of MHC class II genes (81-83), were found at positions -186, -163 and -62, respectively. A sequence (nt -154 to -125), overlapping the X-box-like motif of the human pIgR gene, was identified which is homologous to a sequence found in the promoter region of the bovine cytokeratin IV gene (129). In addition, a potential AP1 binding site (aGAGTCA), similar to the AP1/TRE consensus motif (TGAGTCA), is found at position -76 (130).
Discussion

The polymeric immunoglobulin receptor transports pIg and is also the precursor protein for secretory component, a vital part of the secretory immunoglobulin molecule. As such, regulation of the expression of this receptor may play a crucial role in mucosal immune defense. To begin to examine the molecular events involved in the induction of pIgR expression, the promoter proximal region within the 5'-flanking sequences of the human pIgR gene has been localized and sequenced.

Apparent similarities in expression have led us to suggest that the pIgR and MHC class II antigen genes may be coordinately regulated in mucosal epithelial cells (43, 67; see also Chapter 4). Analysis of the sequence of the promoter proximal region of the pIgR gene has uncovered similarities with the MHC class II genes. The pIgR and MHC class II genes are members of the immunoglobulin gene superfamily (44). This observation points out the possibility that some immunoglobulin superfamily genes may have retained not just structural similarities, but also regulatory features of the ancestral gene(s) from which they evolved.

A single transcriptional start site was demonstrated for the pIgR gene in human intestinal epithelial cells. A classic TATA-box consensus sequence was not found in the expected position upstream of the pIgR start site. However, a sequence was found at position -28 (TTTAA) which may represent a TATA variant
(Fig. 5.2B). Several of the MHC class II genes do not contain TATA sequences and there is evidence that these sequences may not control the proper initiation of transcription of the class II genes (82, 83). It has been speculated that the conserved X- and Y-box sequences, found in all functional MHC class II genes, may specify the start site of transcription. Both X- and Y-box-like sequences have been identified in the promoter proximal region of the human pIgR gene (Figs. 5.2B and 5.3). It is possible that these DNA elements may be involved in selection of the start site of transcription of the pIgR gene. The X-box-like element found in the pIgR gene contains four nucleotides which differ from the MHC class II gene X-box consensus sequence (Fig. 5.3), however it is not unusual for X boxes to vary from the consensus sequence by four or even five nucleotides (83).

The X- and Y-box-like elements in the 5'-flanking region of the pIgR gene may be important clues to the mechanisms of induction of the pIgR gene by cytokines. Induction of the MHC class II genes by IFN-γ has been shown to require the coordinate action of three DNA elements (the X box, the Y box, and the Z/W region, see Fig. 1.4) (81-83). The Z/W region, which lies upstream of the X box in MHC class II genes, consists of a pyrimidine-rich tract called the PY box and a conserved septamer (GAACCTT), the S box. The distance between the S and X boxes is usually conserved (15-17 nt) (131). An S-box-like sequence (GaAgCTg) is found at the correct position (-186) in the human pIgR gene. This
Figure 5.3. Comparison of the S, X and Y box sequences of the promoter proximal regions of the human pIgR and MHC class II genes. Sequences lying between the S, X and Y boxes have been included for the HLA-DRα gene to illustrate the typical conserved spacing of these motifs (15-17 nt between the S and X boxes; 18-20 nt, 2 helical turns, between the X and Y boxes). The spacing between the X- and Y-like boxes of the human pIgR gene is different (87 nt, approximately 9 helical turns). The consensus sequences are the conserved S, X and Y boxes of the MHC class II genes. Nucleotides deviating from the consensus sequences are underlined.
finding supports the possibility that the mechanism of induction of the pIgR and MHC class II genes by IFN-γ may be similar. Although a pyrimidine-rich stretch of 6 or 7 nucleotides (PY box) is not evident, three out of four of the nucleotides which lie directly upstream of the X-box-like element of the pIgR gene are pyrimidines (Fig. 5.2B). Even this small stretch of pyrimidines may function like a PY box to stabilize the binding of proteins to the X box (81). There is one striking difference between the X-Y-box regions of the pIgR and MHC class II genes. The spacing between the X and Y boxes of the MHC class II genes is highly conserved (18-20 nt, 2 helical turns). This spacing is not conserved in the pIgR gene, where the X- and Y-like boxes are separated by 87 nucleotides. However, it is not the precise spacing but rather the alignment of these DNA elements that has been shown to be necessary for their function (131, 132). Although the spacing of these sequences differs between the pIgR and MHC class II genes, the additional 7 helical turns between the X- and Y-like boxes in the pIgR gene may still preserve the stereospecific alignment of these elements.

The idea that these X- and Y-box-like motifs may also be involved in regulation of the pIgR gene by cytokines other than IFN-γ is supported by two recent reports. One of these reports indicated that TNF-α, another cytokine which has also been shown to regulate pIgR and MHC class II genes in coordinate fashion (67), regulates MHC class II gene expression via X-box region
sequences (133). The second report demonstrated the involvement of a Y-box-like sequence in regulation of the expression of human Ig-heavy chain germline transcripts by IL-4 (134), another cytokine that has been shown to regulate the expression of pIgR (68, 74).

Mechanisms for tissue specific expression differ among the MHC class II genes. The DRα gene contains a B-lymphocyte specific octamer motif (135). In other MHC class II genes, a B-cell-specific DNA motif has been demonstrated which lies between positions -1000 and -2000 upstream of the transcriptional start site (83). As expected, B-cell-specific regulatory elements were not evident in the promoter proximal region of the pIgR gene. A regulatory X2 box region, overlapping the 3' end of the X box, has recently been identified in MHC class II genes which has homology to cAMP response elements (CREs) in some class II genes and phorbol ester response elements (TREs) in others (83). It has been suggested that regulatory proteins binding to the X2 box may interact with proteins bound to the X box and may promote transcription by stabilizing the interactions at this locus (136). While no CRE- or TRE-like DNA elements were found overlapping the X box, a sequence was found with homology to the promoter region of the bovine cytokeratin IV gene which occupies the X2-box position in the human pIgR gene (129). The AAPuCCAAA motif located at the 3' end of this sequence in the pIgR gene has been identified in the promoter regions of many
other human, murine and bovine cytokeratin genes. Like pIgR,
keratin proteins are expressed primarily in epithelial cells. It is
interesting to speculate that the binding of epithelial cell-specific
regulatory proteins to this region of the pIgR gene may contribute
to the tissue specificity of pIgR expression. Cell type-specific
proteins might bind to this site and stabilize the binding of factors
with a more ubiquitous tissue distribution to the pIgR X-box-like
DNA element.

Analysis of the sequence of the promoter proximal region of
the human pIgR gene has allowed the identification of several
potential cis-acting regulatory elements, some of which have been
previously demonstrated in the MHC class II genes. Experiments
addressing the functional importance of these sequences in pIgR
expression are already underway in our laboratory. Examination
of the mechanisms involved in epithelial cell-specific induction of
pIgR expression may provide insight into the complex
mechanisms which regulate the transcription of the MHC class II
genes, or may reveal completely new mechanisms for the
regulation of gene expression.
CHAPTER 6

DISCUSSION
Discussion

A distinct immune system protects mucosal surfaces of the body from constant challenges mounted by the external environment. The main effector molecules of this system are the secretory immunoglobulins, especially secretory IgA (8,17). Secretory IgA antibodies are produced at sites of infection by the collaborative effort of plasma cells which produce IgA, and epithelial cells (8, 9, 17, 84). Epithelial cells express the polymeric immunoglobulin receptor, which is composed of Ig-like domains (39). At the basolateral surface of epithelial cells, pIgR binds dIgA via a conserved region of its first Ig-like extracellular domain (45, 46). A disulfide bridge between dIgA and the fifth Ig-like domain of pIgR stabilizes the union between pIgR and dIgA (84, 137). After transcytosis, the pIgR is cleaved and secretory immunoglobulin is released into secretions (37). The ectoplastic portion of the receptor, called secretory component, remains bound to the immunoglobulin and may protect it from degradation in the harsh mucosal environment (13-15). As pIgR does not recycle, regulation of pIgR production may play a role in the regulation of mucosal immune responses (36). The pIgR genomic and cDNAs described in this thesis will provide important tools for future analysis of pIgR function and regulation, and may prove to be useful therapeutically in the control and prevention of human disease.
A primary mode of protection by sIgA is immune exclusion (18). By cross-linking and aggregating pathogens, sIgA can restrict their movement and access to the epithelium until they can be eliminated by mechanical clearance mechanisms. It has been suggested that the low levels of sIgA found in the external secretions of symptomatic AIDS patients may contribute to the frequent mucosal infections seen in these patients (138). Recent experiments performed in mice have shown that monoclonal sIgA alone, if administered in sufficiently large amounts, can provide antigen-specific protection at mucosal sites (17, 139). Administration of monoclonal IgA antibodies that did not contain secretory component also provided protection but this protection was of much shorter duration, possibly due to degradation of the IgA by mucosal proteases. These experiments suggest that passive oral administration of engineered antigen-specific sIgAs might be used therapeutically to combat infection. Passively administered sIgAs might be used in treatment or prevention of infection in patients with either genetically inherited or acquired immunodeficiencies.

To afford maximum protection from degradation in the mucosal environment, sIgAs for passive administration should consist of dIgA and secretory component that have been covalently linked. A co-culture strategy has already been developed to produce this sIgA (17, 139). When epithelial cells transfected with pIgR cDNA are grown on collagen layers in which
IgA-producing hybridoma cells are embedded, disulfide-bonded monoclonal sIgA can be purified from culture medium collected from the apical surface of the epithelial cell layer. Previously, these experiments were performed using the rabbit pIgR cDNA isolated by Mostov et al. (39). The human pIgR cDNA described in this thesis is now being used to transfec epithelial cells for coculture. The sIgA produced by this system will contain human SC and mouse monoclonal antibodies will be "humanized" by substitution of human IgA constant region domains for mouse domains.

It may also be possible to produce covalently linked sIgA in vitro without using a co-culture system. Large amounts of secretory component might be produced by apical secretion from epithelial cells which have been transfected with mutant human pIgR cDNA, lacking the transmembrane and cytoplasmic domains. This deletion has been previously shown to produce a receptor that is secreted predominantly apically (140). The resultant free secretory component might then be chemically linked to monoclonal IgA. Unfortunately, the critical cysteine residues involved in formation of the disulfide bond between pIgR and SC have not yet been identified (114). Site-directed mutagenesis experiments are currently being performed by Chintalacharuvu et al. (personal communication) using the human pIgR cDNA to identify cysteine residues involved in this bond. It is my hope
that studies involving the human plgR cDNA will eventually prove useful in mitigation and prevention of human disease.

Primary polymeric immunoglobulin receptor deficiency has never been convincingly demonstrated (42). The function of plgR in the transport and protection of pIg at mucosal sites may be so important that its absence is incompatible with life. It is also possible that plgR may be merely linked to another life sustaining gene. A close linkage has been observed between plgR and the complement action regulation cluster at 1q32 (141). Using the mouse plgR cDNA described in this thesis, our laboratory has isolated and characterized mouse plgR genomic DNAs. These DNAs are being used to construct a homologous recombination targeting vector for generation of a line of mice devoid of plgR. The study of these mice should provide valuable information on the importance of plgR and sIg in the mucosal immune system. Novel functions for secretory component may also be revealed.

PlgR can undergo transcytosis, and can be cleaved and released as free SC (36, 37). In some secretions, like amniotic fluid, fSC is the predominant form of SC (142). Hirt et al. have demonstrated that phosphorylation of Ser664 in the cytoplasmic region of rabbit plgR is required for entry of the receptor into the transcytotic pathway in the absence but not in the presence of IgA (52). These findings suggest that production of free SC may be regulated differently than production of SC that is bound to IgA (sIgA), which may imply that fSC has its own distinct
function. In addition to mice devoid of pIgR, mice are currently under construction which will carry truncated pIgR genes, without transmembrane or cytoplasmic domains. These mice should be capable of fSC production but should not produce sIg. By also constructing mice which carry murine pIgR that has been altered by site-directed mutagenesis at Ser\textsuperscript{680} (which corresponds to Ser\textsuperscript{664} of rabbit pIgR), it may be possible to engineer mice which produce sIg but not fSC. These mouse model systems might be used to determine if SC has a function apart from its role in the transport and protection of sIg.

Cytokines may direct the mucosal immune response at sites of infection or inflammation by acting locally on the cells of the epithelium to increase the production of pIgR. IFN-\(\gamma\), TNF-\(\alpha\) and IL-4 have all been shown to increase the expression of pIgR \textit{in vitro} in the human intestinal cell-line HT-29 (65-68, 74). Enhancement of pIgR expression by IFN-\(\gamma\) in these cells has been shown to be accompanied by increases in the steady state concentration of pIgR mRNA (43, 70). The only reported demonstration of effects on pIgR expression \textit{in vivo} by cytokines is by Wira et al. (119). They reported that direct application of IFN-\(\gamma\) to the lumen of the rat uterus resulted in an increase in levels of fSC in uterine secretions. As fSC production may be regulated differently than increases in pIgR expression, the effects of IFN-\(\gamma\) and of other cytokines on pIgR expression \textit{in vivo} remain to be elucidated.
Isolation of the mouse pIgR cDNA has made available important tools which can be used for investigation of pIgR regulation \textit{in vivo}. Expression of mouse pIgR protein from this cDNA will allow production of antibodies specific for mouse pIgR/SC. The mouse cDNA has already been used as a probe to examine the tissue specificity of pIgR expression (see Fig. 3.4). This cDNA will provide probes for other Northern blot, nuclease protection and \textit{in situ} hybridization analyses.

The importance of cytokines in pIgR expression might also be studied \textit{in vivo} using normal, germ-free and athymic nude mice. Germ-free mice have recently been used to demonstrate that induction of MHC class II expression on intestinal epithelial cells is dependent on microbial colonization (143). This observation suggests the importance of cytokines on MHC class II expression \textit{in vivo}. If cytokine production is important in pIgR expression, germ-free mice should exhibit decreased pIgR expression compared to littermates that have been removed from their sterile environment. Athymic mice might be expected to demonstrate only low levels of pIgR expression which might be experimentally augmented by co-administration of antigen and syngeneic antigen-specific T cells. Recently, Raz \textit{et al.} have injected normal mice intramuscularly with cDNA expression vectors encoding cytokines and observed the effects of these cytokines on immune responses \textit{in vivo} (144). It may be possible
to observe the effects of cytokines on pIgR expression using this system.

The findings presented in this thesis have provided insight into possible mechanisms of regulation of pIgR expression in epithelial cells. The induction of pIgR expression in epithelial cells by IFN-γ involves increases in pIgR mRNA that are protein synthesis dependent (Chapter 4). The mechanism of pIgR induction may include the *de novo* synthesis of one or more transcription factors which bind to DNA response elements in the pIgR gene. The 5'-flanking region of the pIgR gene, isolated as a part of this thesis work, was shown to contain sequence elements that are similar to important control elements found in the promoter proximal regions of MHC class II genes. A series of deletions of the 5'-flanking sequence of the pIgR gene have already been placed into vectors bearing either the luciferase or the chloramphenicol acetyltransferase reporter gene. In future experiments, these recombinant plasmids will be used in transient transfection assays to address the function of pIgR flanking region sequences in induction of receptor expression by IFN-γ and other cytokines which regulate pIgR. Gel mobility-shift DNA-binding assays, performed in our laboratory, have demonstrated the presence of proteins which bind to MHC class II X- and Y-box sequences in nuclear extracts from human epithelial cells (145). In the future, binding assays can be performed using DNA fragments containing the native X- and Y-box-like motifs of the
pIgR gene. Antibodies which are specific for MHC class II X- and Y-box binding proteins (81) could be used in binding assays to assess the relationship of proteins which bind to the X- and Y-box-like motifs of the pIgR gene with proteins that bind similar elements in MHC class II genes. These experiments might also provide information that may be beneficial in understanding the complex mechanisms which regulate the transcription of the MHC class II genes.

In addition, future studies of pIgR regulation might include examination of the mechanisms involved in tissue specificity of pIgR expression. As pIgR is expressed on hepatocytes in rodents but not on human hepatocytes (55, 57), it may prove interesting to explore species-specific differences in the tissue specificity of pIgR expression. The isolation of the mouse pIgR cDNA has already facilitated identification of mouse pIgR genomic DNAs by our laboratory. It is probable that these DNAs contain the 5' flanking sequences of the mouse pIgR gene. Their characterization will allow comparison of the promoter proximal regions of the human and mouse pIgR genes. Sequence comparisons and DNA-binding assays could be used to reveal whether it is a divergence in sequence or in a DNA-binding activity that is the basis of the species-specific difference in hepatocytic pIgR expression. Availability of the 5'-flanking region of the mouse pIgR gene would also allow study of the tissue
specificity of pIgR expression in transgenic mice where this sequence could be linked to a reporter gene.

IFN-γ has been shown to regulate human gene expression both transcriptionally and post-transcriptionally (80). In this thesis study, IFN-γ-induced increases in pIgR mRNA required 12 h (Fig. 4.4) and were sensitive to a block in protein synthesis at any time during this period (Fig. 4.5). These observations suggest that pIgR mRNA accumulation may occur by a cascade of events, one or more of which may require de novo protein synthesis. Nuclear runoff assays are currently being planned in our laboratory to measure changes in the rate of transcription of the pIgR gene in response to IFN-γ. These experiments will allow an assessment of the role played by newly synthesized transcription factors in pIgR mRNA induction by IFN-γ.

Another consideration must be that pIgR mRNA accumulation may occur by the action of newly synthesized proteins which act to increase the stability of pIgR mRNA. The primary determinants of eukaryotic messenger RNA stability are the structure of the molecule and its interaction with cytoplasmic protein(s) (146). An AUUUA sequence motif has been found in the 3'-UTRs of several unstable mRNAs, and a cytoplasmic protein has been recognized which binds to this sequence making these RNAs susceptible to degradation (146). An AUUUA sequence is located directly upstream of the poly(A) addition sequence in the rabbit pIgR mRNA (39). This sequence motif is not present in the
3'-UTRs of pIgR cDNAs that have been reported for mouse (see Fig. 3.1), human (42, 43; see also Fig. 3.2) or rat (40). However, the cDNAs reported for these species contain 3'-UTR sequences which lack poly(A) addition motifs and are all considerably shorter than the 3'-UTR reported for rabbit pIgR (676, 443, 700 and 1072 nt for the mouse, human, rat and rabbit 3'-UTRs, respectively). In addition, discrepancies observed in the estimated sizes of these mRNAs from Northern blots suggest that the 3'-UTR sequences reported for the mouse, human and rat may all be incomplete. Using PCR techniques, it may be possible to recover the full-length 3'-UTR sequences of these pIgR mRNAs for analysis. The presence of an AUUUA motif may be a common feature of all pIgR mRNAs. It is possible that IFN-γ may allow the accumulation of pIgR mRNA by mechanisms which include the de novo synthesis of a protein which stabilizes an inherently unstable pIgR mRNA. Alternatively, the 3'-UTR of pIgR might assume a three-dimensional structure that is important in regulation of the stability of pIgR mRNA. In the future, studies of the structure of the pIgR mRNA and of the regulatory mechanisms involved in its turnover may help to expand current knowledge of the molecular basis of this important aspect of gene expression.
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