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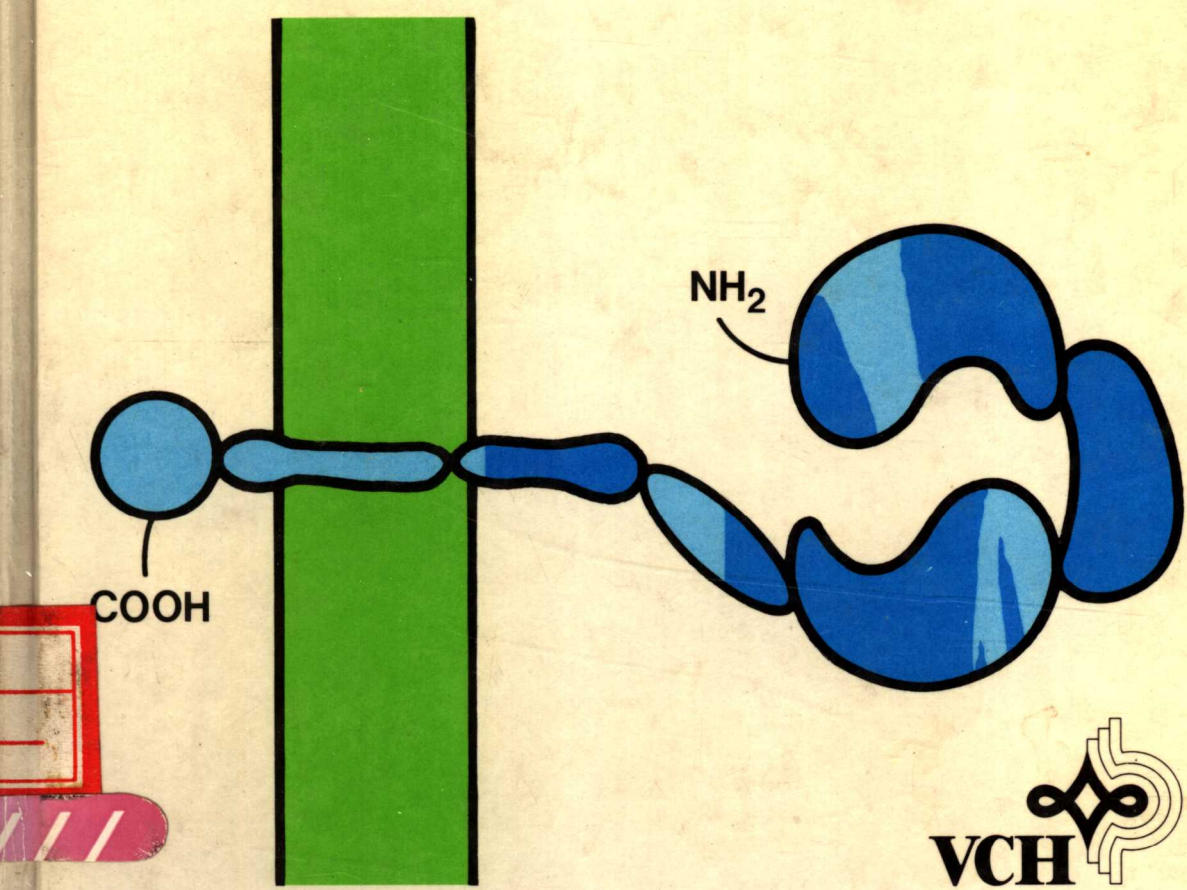
The Molecular Biology of Receptors

Techniques and Applications of Receptor Research

Edited by A. D. Strosberg



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This timely description of receptor research, stemming from an increased understanding of membrane receptor functions, shows how modern receptor technology utilizes methods from molecular biology and gene cloning. It is a valuable investigation of how receptors receive extracellular signals and thereupon generate transmembrane and intracellular signals which result in specific biological responses. The complete understanding of this process is the goal of receptor research. This collection of studies by the world's leading specialists will help scientists to approach that goal.

Presenting the most advanced techniques in an applied setting, the authors demonstrate their application to a wide variety of problems. Scientists worldwide involved in receptor research will find in this book an important state-of-the-art review and a lasting reference source. It will also guide them in developing an approach to the study of receptor systems other than those described here.

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Abbreviations

ADF	adult T-cell leukemia derived factor
ATL	adult T-cell leukemia
BSA	bovine serumalbumine
cDNA	complementary DNA
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate
CHO	carbohydrate
CM	conditioned medium
CNS	central nervous system
Con A	concanavalin A
Da	daltons
DTT	dithiothreitol
EGF	epidermal growth factor
FSH	follicle stimulating hormone
GABA	γ-aminobutyric acid
GDP	guanosine triphosphate
GH	growth hormone
GlcNAc	*N*-acetyl glucosamine
Gly R	glycine receptor
GTP	guanosine triphosphate
HPLC	high pressure liquid chromatography
HTLV-I	human T lymphotropic virus-I
IGF	insulin-like growth factor
Il-1,2	interleukin 1,2
Il-2R	interleukin 2 receptor
kb	kilo base
kDa	kilo daltons
LDL	low density lipoprotein
LH	luteinizing hormone
mAb	monoclonal antibody

mRNA	messenger RNA
NGF	nerve growth factor
PBS	phosphate buffered saline
PBL	peripheral blood lymphocyte
PEG	polyethylene glycol
PNA	peanut agglutinin
PRL	prolactin
RIA	radio immuno assay
RPMI	Roswell Park Memorial Institute
RRA	receptor radio assay
SDS	sodium dodecyl sulfate
Tac antigen	antigen present on activated T-cells
TGF	tumor growth factor
VVGF	vaccin virus growth factor

Introduction

The present volume stems from the realization that our understanding of how membrane receptors work has progressed considerably through the application of molecular biological techniques to the elucidation of the amino-acid sequence of these elusive proteins. We have assembled in this book a number of practical discussions concerning the study of various plasma membrane receptors. Some of these investigations have progressed to the analysis of the primary structures whereas others still are hampered by difficulties which the authors expose in detail. The receptors discussed here differ considerably or by their structure and function, and various strategies have been elaborated to perform their analysis.

A. A COMMON STRATEGY: FROM PROTEIN TO DNA TO PROTEIN

This strategy has been used for several receptors discussed in this book, and encompasses the following steps:

1. Solubilization of membranes which contain receptors.
2. Affinity chromatography or immunoaffinity of receptors.
3. Further purification of receptors through additional procedures such as preparative electrophoresis or HPLC.
4. Amino-acid microsequencing of amino-terminal or other portions of receptors.
5. Synthesis of oligonucleotides corresponding to the amino-acid sequences.
6. Identification of cDNA or genomic clones coding for receptors by DNA/DNA hybridization with the synthetic probes.
7. Nucleotide sequence determination of the clones reacting with at least two non-overlapping synthetic oligonucleotides.
8. Preparation of antibodies against peptides predicted from nucleotide sequence and analysis of reactivity towards natural receptors.
9. Expression of receptor mRNA or gene and functional analysis of the product.

B. ALTERNATIVE APPROACHES

1. Subtractive hybridization

The gene coding for the receptor for antigen on T lymphocytes was identified by applying subtractive hybridization of cDNA clones. This was achieved by analysing only those T-cell-specific cDNA clones which were not recognized by RNA prepared from B lymphocytes or from other types of cells. Among the antigen-specific T-cell cDNA clones, only those which showed rearrangement when compared to embryonic genomic DNA were retained for further studies. These investigations revealed that the genes coding for the α, β, γ and δ chains of T-cell receptors for antigen display an organization quite similar to that of the immunoglobulin genes in B cells: variable (V), diversity (D) only found in the β chains so far, joining (J) and constant (C) segments all separated by introns in genomic DNA are rearranged to form an active VDJC complex in the antigen-responsive cell (for the most recent short review, see ref [1]).

2. SCREENING AND CLONING IN EXPRESSION LIBRARIES OR TRANSFECTED CELLS

Several λ bacteriophage-derived vectors have been developed which favour expression of the adjacent gene in bacteria. One of these, λgt11, has been used by several groups to detect products of receptor genes by antibodies raised against the receptor proteins. Procedures to obtain such anti-receptor antibodies range from screening sera from patients with auto-immune diseases, to immunization with purified receptor or anti-ligand antibody to induce antiidiotypic antibody specific for receptor.

This type of approach was used for the cloning of the T8 (now CD8) antigen of T lymphocytes which is thought to act as a receptor for class II histocompatibility antigen. Its gene was cloned by transfer of human genomic DNA together with a thymidine kinase gene, into tk^- mouse L cells. Co-transformants now expressing the T8 antigen were detected with anti-T8 antibody; cDNA synthetized from the mRNA of the transformants was hybridized first to an excess of mRNA from non-transformed L cells, then used to screen libraries of T8 and T8-negative human peripheral T cells [2].

3. SCREENING OF GENOMIC LIBRARIES WITH RECEPTOR GENE PROBES

As discussed at greater length in Chapter 1 by Gullick and Waterfield, the EGF receptor displays a number of structural properties (cysteine-rich clusters, hydrophobic transmembrane domain, kinase domain) which have been subsequently found in several other growth factor binding proteins, including the insulin receptor, (see Chapter 2 by Ullrich and Ramachandran) as well as in precursors of such factors, and in oncogenes.

Using fragments of the EGF receptor gene as probes, several homologous genes

have been identified: in man, one would code for a receptor with extensive homology to the EGF receptor [3].

The analysis of DNA-derived sequences of the precursors of EGF, of transforming growth factor (TGF-α) and vaccinia virus p19 has revealed the presence of hydrophobic sequences similar to the transmembrane domains found in most cell surface receptors. More strikingly, the EGF precursor shares extensive homology with the low-density lipoprotein receptor, suggesting that in its unprocessed form, this precursor may act as a cell surface receptor of yet unknown biological function [4].

C. PERSPECTIVES

Although the knowledge of the sequences and the availability of the corresponding genes expressed in suitable cells constitute important milestones in receptor research, it has already become obvious that they actually provide considerable opportunity for further molecular investigations. These are directed towards several goals:

- the determination of the folding of the polypeptide chain and the localization of the various functional domains and binding sites,
- the understanding of how receptors function in their membrane environment and interact with other molecules which are connected to the cytoskeleton, which mediate effector function, receptor movement or turn-over,
- the elucidation of structure and function of the various second-messenger systems triggered by the receptor–agonist interaction,
- the regulation of receptor expression and desensitization,
- the discovery of new receptors which are either structurally homologous or/and constitute pharmacologic subtypes,
- the isolation of new endogenous ligands, hormones, neurotransmitter or growth factors by the use of receptor-affinity gels.

These exciting new directions will immensely benefit from some of the *molecular biology-based directed mutagenesis* procedures so well applied to two receptor types not discussed in this volume, namely the nicotinic acetylcholine receptor and the steroid receptors.

The *immunologic approach* will also contribute decisively to the progress of the knowledge of the topology of receptors embedded in membranes as well as to the comparison of similar receptors for different tissues and organs or different species.

Last but not least, the *pharmacologic studies* should continue to lead the way in the fine analysis of receptor specificity. The recent confirmation by Numa and his coworkers of distinct muscarinic acetylcholine receptor genes coding respectively for M_1 (high affinity for pirenzepine [5]) and M_2 (low affinity for pirenzepine [6]) subtypes does indeed remind us of the remarkable power of binding studies. Much interest of the medical community is now directed towards the discovery or synthesis of antagonists to various tumour growth factors and new natural ligands for various neuroreceptors.

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1

Epidermal growth factor and its receptor

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1. INTRODUCTION

The Epidermal Growth Factor (EGF) has been a subject of study for more than twenty-five years, and during that time a large body of knowledge has accumulated about its structure and function. The receptor for EGF was identified more than ten years ago, and since then much information has been published concerning its distribution, structure and enzyme activity.

In this chapter we will firstly consider EGF and the more recently discovered related growth factors which now form the EGF family. Secondly, we shall review some aspects of the normal expression and activity of the EGF receptor. We will not be discussing the relationship of the EGF receptor to the retrovirally encoded oncogene product, v-erbB, or aberrant receptor expression in human malignancies, since these topics are sufficient for a chapter of their own.

2. THE EPIDERMAL GROWTH FACTOR FAMILY

2.1 Epidermal growth factor

During the early 1960s, Stanley Cohen at Vanderbilt University worked on the nerve growth promoting activity of mouse submaxillary gland, and discovered that partially purified preparations contained an additional biologically active component [1]. This material was purified by a series of ion exchange and gel filtration steps using as an assay its effect of stimulating precocious eyelid opening in newborn mice. Initially called 'Tooth-lid Factor', its name was subsequently changed to Epidermal

Growth Factor (EGF). In a series of papers, Cohen and his collaborators described a more rapid and efficient method of purification [2], which allowed the complete sequence of the protein to be determined [3]. The molecule is a 53 amino acid polypeptide of molecular weight 6045 daltons, lacking phenylalanine, lysine and alanine, but containing six cysteine residues arranged in three disulphide bonds [3], the positions of which are known (Fig. 1). The structural motif of cysteine residues is

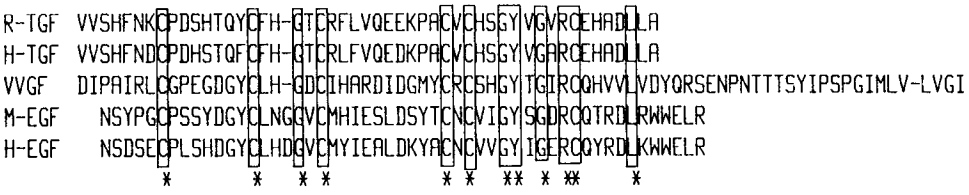

Fig. 1 — The sequences of mouse EGF (M-EGF), human EGF/urogastrone (H-EGF), rat TGFα (R-TGF), human TGFα (H-TGF) and vaccinia virus growth factor (VVGF) aligned for maximal homology. The asterisks indicate the position of the conserved cysteine residues. Residues conserved between all five molecules are boxed.

the central paradigm for all the subsequently discovered members of the EGF family of factors, and has been found in molecules as diverse as the LDL receptor and homeotic genes of a nematode and *Drosophila.* The structure of the protein precursor of this 53 amino acid polypeptide has been determined by cDNA cloning which revealed the completely unexpected discovery that it was initially synthesized from a 4.7 kb mRNA as a 1168 amino acid precursor protein [4]. Equally surprising was that the six cysteine residue motif was repeated with minor variations of spacing eight times within the precursor molecule. Whether any of these additional structures are biologically relevant remains to be determined. Some high molecular weight immunoreactive forms of EGF have been reported, presumably as a consequence of differential proteolytic processing [5].

The distribution of EGF protein and EGF mRNA in normal tissues has been studied by immunostaining and Northern hybridization. These studies have been almost as baffling as they have been revealing. By far the richest source of EGF discovered to date is the adult male mouse submaxillary gland which is rich both in EGF protein [6] and in EGF mRNA [6,7]. Considerable quantities of pre-pro EGF mRNA were also found in mouse kidney, although there was little detectable protein. All other mouse tissues, excepting liver, eye and L cells, contained much lower but measurable quantities of mRNA.

The human cognate of mouse EGF was isolated in 1974 by Gregory and Willshire (U.K. Patent Specification No. 1394846) in attempts to define an anti-gastric acid secretory factor which had previously been called urogastrone. Urogastrone is now an accepted name for human EGF. The sequence of the molecule was determined

and found to be composed of 53 amino acids, of which 37 were homologous in mouse F [8], including the now characteristically spaced cysteine residues. Despite the 165 amino acid differences, reflected in the fact that antisera to each molecule do not crossreact, the biological effects of mouse EGF and human EGF/urogastrone are indistinguishable [9]. Recently, the structures of EGFs from several other species have been determined [10].

The tissue distribution of human EGF protein has been examined by immunofluorescent staining. The glands of Brunner in the intestine and some cells in the ducts of the submandibular salivary glands were positive. All other tissues studied failed to reveal any fluorescence above background [11]. Subsequently, there has been a report of EGF immunoreactive material in the CNS, particularly in the forebrain and midbrain structures of pallidal areas [12]. In this work, the authors speculate that EGF may represent a 'gut–brain' peptide with potential neurotransmitter–neuromodulator functions.

There has been some controversy as to the levels of EGF present in the blood. Bowen-Pope and Ross have reported data that go some way to clarifying this point in which they emphasize that the method of measurement, RIA or RRA, can influence the apparent concentration determined. Present estimates range from about 0.2 to 2.0 ng/ml in human plasma [13,14] which, assuming the 53 amino acid form, would represent concentrations from 3 to 30 $\times$ 10^{-11}M. However, there is some evidence that forms of EGF may be found in platelets [5] which, from previous experience with PDGF, suggests that serum levels may not necessarily be representative of *in vivo* plasma concentrations.

Although a rich source of biologically active EGF exists in the mouse submaxillary gland, providing enough material suitable for research purposes, alternative methods of large-scale production have been encouraged because of the finding that injection of EGF into sheep cause shedding of wool [15]. The EGF gene has now been cloned and expressed in yeast [16] and in *E. coli* [17], and has been chemically synthesized by the solid phase method [18]. The gene for EGF has been localized to chromosome 4 in humans [19] and chromosome 3 in mice [20] using cloned DNA probes.

2.2 Transforming growth factor, type α (TGFα)

In 1978, DeLarco and Todaro discovered that mouse fibroblasts transformed by murine sarcoma viruses produced and excreted polypeptide growth factors which competed for binding with ^{125}I EGF to EGF receptors, but which were antigenically distinct from mouse EGF [21]. Subsequently, many reports appeared of similar factors produced by a wide variety of cells transformed by viruses and chemicals and in many human tumour cell lines [22,23]. Further purification of the material originally called sarcoma growth factor revealed that it was separable into two components, one of which was named TGFα that competed for EGF binding and stimulated the growth of small colonies of indicator cells (NRK, clone 49) in soft agar, and another factor called TGFβ which did not prevent EGF binding and produced larger colonies [24]. TGFα mRNA has been detected in extracts of whole mouse embryos with a peak appearing at day 7 and a smaller peak at day 13 [25], and during foetal rat development at days 8–10 which then declined to undetectable