Creation of the Immunosensor Protein (Artificial Receptor) Whose Protein Kinase Activity Can Respond to Antigen

(抗原により蛋白質リン酸化活性の変化する免疫センサー蛋白質(人工受容体)の創製)

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Creation of the Immunosensor Protein (Artificial Receptor) Whose Protein Kinase Activity Can Respond to Antigen

Hiroshi Ueda

Department of Chemical Engineering
Faculty of Engineering, The University of Tokyo
7-3-1 Hongo, Bunkyo-ku, Tokyo 113

Phone/Fax: 03-5800-6800

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Abbreviations

aa	amino acids
Ab	antibody
bp	base pairs
BPB	bromophenol blue
BSA	bovine serum albumin
cap	6-aminohexanic acid
ccc	covalently closed circular
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acids
EDTA	ethylenediaminetetraacetic acid
EGTA	ethyleneglycol bis(2-aminoethylether)tetraacetic acid
ELISA	enzyme-linked immunoadsorbent assay
ER	endoplasmic reticulum
FCS	fetal calf serum
FITC	fluoresceine-isothiocyanate
HRP	horseradish peroxidase
hrs	hours
Ig	immunoglobulin
IL	interleukin
kD	kilodalton
2ME	2-mercaptoethanol
MEM	modified Eagle's medium
NP	4-hydroxy-3-nitrophenylacetic acid
PBS	phosphate-buffered saline
PEG	polyethyleneglycol
PMSF	phenylmethanesulfonyl fluoride
PtdIns	phosphatidylinositol
RER	rough endoplasmic reticulum
rpm	round per minute
SER	smooth endoplasmic reticulum
SDS	sodium dodecyl sulfate
Tris	Tris(hydroxymethyl)aminomethane
W.T.	wild type

Abbreviations for amino acids in one letter code

Alanine	A
Arginine	R
Aspargine	N
Cysteine	C
Glutamine	Q
Glutamate	E
Glycine	G
Histidine	H
Isoleucine	I
Leucine	L
Lysine	K
Methionine	M
Phenylalanine	F
Proline	P
Serine	S
Threonine	T
Tryptophane	W
Tyrosine	Y
Valine	V

Chapter 1 Introduction

1.1. Overview of this research

The biosensor which utilizes immunoglobulin, so called immunosensor, has been a fascinating research target because of its high specificity and wide applicability. The immunosensor consists of antigen-recognition portion (antibody) and signal amplifier portion which amplifies antigen-binding signal. As this signal amplifier, a variety of methods are proposed: a method using complement and liposome, a method using electrochemical luminescence, etc. However, these methods have disadvantage to the ordinary time-consuming immunoassay such as ELISA in sensitivity and stability since (in most case) it is very difficult to determine the amount of the bound antigen in the presence of the background of other non-specific binding protein. However if the immunoglobulin has an enzymatic activity which can be activated by antigen, the antigen binding signal may be easily detected and a very sensitive and stable sensor will be realized.

As methods of providing an enzymatic activity to antibody, chemically crosslinked antibody-enzyme conjugates such as Ab-horseradish peroxidase have been made and widely used. But in this method, the regulation of enzymatic activity seems to be quite difficult because the structures of conjugates are heterogeneous. The separation of each conjugate is almost impossible. So as the first phase of this study, elucidation of the conjugation method using genetic engineering and a system to evaluate its function had to be made.

As an enzyme for conjugation, we chose human epidermal growth factor (EGF) receptor. Naturally, the EGF receptor locates on the plasmamembrane of epidermal and fibroblastic cells and has the ligand

(EGF) inducible protein tyrosine kinase (PTK) activity, which induce cell growth. The EGF receptor was reported to retain the ligand inducible PTK activity when its whole ligand binding domain was exchanged to that of human insulin receptor, a related receptor which also possess PTK activity.

On the other hand, immunoglobulin IgM is expressed naturally on the surface of B cell which is a progenitor of immunoglobulin producing cell (plasma cell). Each B cell expresses a specific antigen receptor (IgM, IgD etc.) and when its corresponding antigen binds to the receptor, the B cell begins to proliferate and differentiate to plasma cell. So the relation between antigen and IgM is quite analogous to EGF and EGF receptor with regard to growth signal transduction.

So as the first model conjugate, I made a chimeric protein between immunoglobulin IgM and cytoplasmic domain of EGF receptor. The constructed chimeric receptor was expressed on the plasmamembrane of the cultured cells, which are to be used to detect the antigen molecule present in the supernatant from their growth-related responses such as rise in intracellular calcium ion. The expressed protein itself may be used *in vitro* as a molecular sensor which receives desired molecular signal and transmit signal as phosphorylation.

To make chimeric protein, a chimeric DNA which is human or avian EGFR cytoplasmic region cDNA joined to a hapten specific murine IgM genomic DNA was made together with Ig promoter/ enhancer. The connected fragment was inserted into an eukaryotic/ prokaryotic shuttle vector and then introduced into myeloma cell which produces the light chain.

The expressed chimeric protein had both antigen-binding and protein tyrosine kinase activity. Moreover, despite the cell's intrinsic inability to bring membrane-type IgM to plasmamembrane from rough

endoplasmic reticulum(RER), the chimeric protein was efficiently transported to plasmamembrane.

As the second phase of this study, I focused on this interesting phenomenon . Cell sorting and cloning revealed a very efficient expression of truncated (cytoplasmic-regionless) receptor on plasmamembrane. To analyze the structural requirement of the efficient transport of this protein, several experiments such as making of chimeric protein, analysis of subcellular fractionation, point-mutation and transplantation techniques were done. As a result, it was made clear that acidic linker sequence which locates just N-terminal to membrane IgM transmembrane sequence works inhibitory to transport.

The third phase of this study was devoted to the design and fabrication of the antigen-responsive chimeric receptor, the ultimate goal of this study. It was based on an hypothesis on the working mechanism of EGFR, which has become clearer in recent few years. As a result, a chimeric protein which showed about tenfold increase in *in vitro* antigen-dependent autophosphorylation activity was obtained.

Chapter 2. Previous researches

2.1. Epidermal growth factor (EGF) receptor

2.1.1. Introduction

Since the pioneering discoveries of the nerve growth factor (Levi-Montalcini et al., 1966) and epidermal growth factor (EGF) (Cohen et al.,1962), it has become clear that polypeptide growth factors play a crucial role in the proliferation, survival, and differentiation of eukaryotic cells. Since then, many new growth factors have been discovered; some of them exert their biological effects on specific cell lineages, while others have broad specificity for many cell types from many tissues (James & Bradshaw, 1984). EGF, because it is the best characterized growth factor, may serve as a model system for exploring the molecular mechanisms underlying mitogenic stimulation. Moreover, it has become clear that specific lesions in the EGF receptor, or abnormal expression of growth factors that mediate their effects by activating the EGF receptors, may be associated with certain cancers. Therefore, the elucidation of the mechanism of action of EGF will certainly provide important clues to fundamental questions concerning the action of growth factors in general and their role in oncogenesis.

2.1.2. Overview of EGF

EGF was isolated from murine submandibular in 1962 by Cohen, as the tooth-lid-factor because it promotes the growth of murine eyelids and teeth. Since he found that these actions derived from the growth of epidermal cell by microscopic analysis, it was later renamed as EGF.

The primary structure of EGF was determined by his group to be composed of 53 a.a. with MW of 6,045(Fig.2-1). Recently, its tertiary structure was obtained by using NMR(Cooke et al., 1987) (Fig.2-2).

While obtained from murine submandilar at first, EGF is contained in almost all body fluid such as blood plasma, urine, milk, saliva, tear, and sweat. In urine it was known as β-urogaston, a factor which represses secretion of acid in stomach. The target of EGF action is very wide and its species specificity is weak. EGF promotes the growth of epidermal cell such as hepatocytes and keratinocytes together with fibroblasts and vascular smooth muscle cells.

Surprisingly EGF is synthesized as a huge transmembrane precursor of MW 130-kD, and it is cleaved to yield a mature EGF of 6-kD. The precursor has 8 more EGF like structures. This EGF-like repeat is observed in many other proteins such as transforming growth factor α, several blood coagulation factors, cell adhesion molecules and low density lipoprotein receptor. However the true physiological function of EGF like repeat is as yet unclear, except that some of these is revealed to bind Ca^{2+} cation.

2.1.3. The structure of the EGF receptor

EGF binds to a transmembrane glycoprotein and activates its protein tyrosine kinase activity(Carpenter and Cohen, 1979). After purification of the human EGF receptor by immunoaffinity chromatography(Yarden et al., 1985) and its partial sequencing (Downward et al., 1984), the complete primary structure of the EGF receptor was deduced from nucleotide sequence of cDNA clones(Ullrich et al., 1984). The following picture of the structure of EGF receptor emerges. The mature receptor is composed of 1186 amino acid residues

that are preceded at the NH_2-terminal end by a signal peptide of 24 hydrophobic amino acids. The signal peptide is cleaved after insertion of the nascent receptor into the membrane of the endoplasmic reticulum. Cotranslationally, the receptor is glycosylated and transported through the Golgi apparatus to the plasma membrane. The mature receptor is composed of three major structural elements(Fig.2-3). The first is an extracellular EGF-binding domain composed of 621 amino acid residues, anchored to the plasma membrane by a single transmembrane region of 23 hydrophobic amino acids. The transmembrane region is followed by a sequence of mostly basic residues; a feature common to many membrane proteins. The cytoplasmic domain of EGF receptor is composed of 542 amino acids. It contains a region of ~300 amino acid residues that is homologous to the catalytic domain of the protein tyrosine kinase encoded by the *src* family of oncogenes(Hunter, 1985). Like the other protein tyrosine kinases, the catalytic domain of EGF receptor kinase contains a lysine(Lys 721) residue (Fig.2-3) that is located 15 residues to the carboxy-terminal side of a consensus sequence, Gly-X-Gly-X-Phe-Gly-X-Val. The lysine residue, together with the consensus sequence, probably functions as part of the ATP binding site(Hunter, 1985).

The binding of EGF to the receptor induces the activation of the protein tyrosine kinase, which phosphorylates various cellular proteins as well as the EGF receptor itself. In intact cells, autophosphorylation occurs mainly on Tyr1173; however, at least two additional tyrosine residues located at the carboxy-terminal end of EGF receptor are phosphorylated when EGF is added to solubilized membranes or to the pure receptor(Hunter, 1985). It was suggested that the autophosphorylation of EGF receptor regulates its capacity to phosphorylate exogenous substrates(Betrics and Gill, 1985).

2.1.4. The EGF receptor and cell transformation

The v-*erbB* oncogene of avian erythroblastosis virus has been shown to encode a truncated EGF receptor and it is proposed that the v-*erbB* protein induces transformation by functioning as an activated growth factor receptor (Downward et al., 1984). The v-*erbB* protein (Yamamoto et al., 1983) is devoid of most of the extracellular domain of EGF receptor and also of 32 amino acid residues at the carboxy-terminal end of the receptor, thus losing the major autophosphorylation site of the native receptor(Fig.2-3). Nevertheless, the v-*erbB* protein possesses intrinsic protein tyrosine kinase activity towards exogenous substrates and also undergoes autophosphorylation (Kris et al., 1985). Chicken erythroblastosis is also caused by avian leukosis virus, which transforms these cells by activating the c-*erbB*/EGF receptor gene through a promoter insertion mechanism. Numerous studies suggest that the EGF receptor may play a role in oncogenesis through an autocline mechanism. Various animal and human tumor cells produce a growth factor called transformation growth factor-alpha (TGFα)(Todaro et al., 1980). This growth factor is a member of the EGF gene family. It binds to cells bearing EGF receptor with an affinity similar to that of EGF and stimulates their proliferation. Finally, it was shown that the EGF receptor gene is amplified and rearranged in many human brain tumors of glial origin(Libermann, 1985). The resultant overexpression of the EGF receptor may play a role in the development or progression of these tumors.

2.1.5. Models for activation of the EGF receptor

An interesting conclusion drawn from the primary structure of EGF receptor(Ullrich et al., 1984) is that the extracellular ligand binding domain is connected to the cytoplasmic kinase region by a single transmembrane region. This is in contrast to other transmembrane proteins that are also involved in signal transduction across the plasma membrane and that usually traverse the lipid bilayer more than once. Two distinct mechanisms can be reasoned for the transmembrane signaling of membrane proteins with a single transmembrane region such as the EGF receptor. In an intramolecular model a vertical dislocation of the membrane hydrophobic stretch of EGF receptor is required for the transfer of a conformational change from the extracellular domain to the cytoplasmic kinase region. However, the juxtamembranal sequence of EGF receptor contains charged amino acids at both faces of the plasma membrane, which may impose a high energy barrier for a putative vertical conformational change from the extracellular domain to the cytoplasmic region(Livneh et al.,1985; Ullrich et al., 1984).

An alternative mechanism involves an intermolecular allosteric process in which ligand-induced receptor oligomerization leads to the activation of the protein tyrosine kinase domain by subunit interaction between neighboring cytoplasmic domains, thus bypassing the requirement for a conformational change to propagate through the transmembrane region(Fig.2-4). Recent results indicate that EGF-induced receptor self-phosphorylation has a parabolic dependence on the concentration of EGF receptor and that cross-linking of EGF receptor by antibodies or lectins stimulates receptor self-phosphorylation. It was also shown that in native gels, EGF receptor migrates in two forms: a fast-migrating form and an EGF-induced slow-migrating form. Based on various control and calibration experiments (Yarden and Schlessinger, 1987) it was concluded that the low form represents the monomeric 170-

kD EGF receptor and the high form represents an EGF receptor dimer. The binding of EGF causes a rapid, temperature-sensitive dimerization of EGF receptors. Receptor dimerization is fully reversible and involves saturable, noncovalent interactions that are stable at neutral pH and in nonionic detergents.

EGF receptor is one of receptor tyrosine kinases such as receptors for platelet-derived growth factor (PDGF), insulin-like growth factor 1(IGF-1), colony-stimulating factor 1(CSF-1) etc., which deliver various signals on the membrane of various cells and tissues. On the basis of sequence similarity and distinct structural characteristics, it is possible to classify many receptor tyrosine kinases into several subclasses(Fig.2-5). Characteristic structural features of the subclasses include two cysteine-rich repeat sequences in the extracellular domain of monomeric subclass I receptors including EGF receptor, disulfide-linked heterotetrameric $\alpha_2\beta_2$ structures with similar cysteine-rich sequences in subclass II receptors, and five or three immunoglobulin-like repeats in the extracellular domains of subclass III and IV receptors, respectively (Ullrich & Schlessinger, 1990).

Receptor oligomerization is an universal phenomenon among growth factor receptors (Fig. 2-6). The universalilty of the dimer-mediated receptor activation mechanism for all receptor tyrosine kinase subclasses and the conservation of the structural parameters involved has been demonstrated by the construction of fully functional chimeric receptors consisting of major domains of different receptor subclasses (Riedel et al., 1989, and references therein). The dimerization mechanism further implies the possible existence of hybrid complexes between structurally very similar receptors such as a and b type PDGF receptors, EGF receptor and HER2/*neu*, or insulin and IGF-1 receptors.

In some cases heterodimer formation has already been demonstrated (Hammacher et al.., 1989; Soos and Siddle, 1989).

2.1.6. Role of autophosphorylation - signal transduction via SH2/SH3 - containing proteins

The most prominent paradox when the cell is stimulated by ligand is that the most intensely tyrosine-phosphorylated protein is the receptor itself. This paradox has been left unsolved for a long time. However, recent findings suggest that this is no longer strange because the autophosphorylation of receptor itself may be important in signal transduction. Wahl et al. (1988) observed an increase of the phosphatidylinositol-specific phospholipase activity in anti-phosphotyrosine purified immunocomplex when the cell which expresses large amount of EGF receptor was stimulated with EGF. Phosphatidylinositol-specific phospholipase (phospholipase C, PLC) activity is thought to be important in cellular activation because one product of PLC diacylglycerol is thought to activate protein kinase C, a Ca^{2+}- dependent threonine/serine kinase which is a key enzyme in cellular activation, and the other product inositol-triphosphate (IP_3) is a ligand for Ca^{2+} channel which release lumenal Ca^{2+} to increase cytoplasmic Ca^{2+} level, one of key events in cellular activation (Nishizuka, 1984). Because the most tyrosine-phosphorylated protein was EGF receptor, a tyrosine-phosphorylation-dependent physical association of EGF receptor with PLC, or tyrosine-phosphorylation-dependent activation of PLC associated with EGF receptor was strongly suggested.

An independent observation which suggested the importance of receptor autophosphorylation was obtained from analysis of *v-crk* oncogene. *V-crk* is a retrovirus-derived oncogene with abnormal

characteristics; while *v-crk* itself has no coding region for tyrosine-kinase activity, the level of tyrosine-phosphorylation of the cell infected with the virus with *v-crk* is extremely high. Instead of tyrosine-kinase, P47*gag-crk* encodes SH2 sequence which is homologous to a region of *src* oncogene. P47*gag-crk* has a remarkable property of forming stable complexes with several tyrosine phosphorylated proteins in *v-crk* -transformed fibroblasts (Mayer et al.,1988; Mayer & Hanafusa, 1990; Matsuda et al.,1990). SH2 domains apparently regulate protein-protein interactions by recognizing peptide sequences that encompass tyrosine phosphorylation sites. Tyrosine phosphorylation of the relevant ligand acts as a switch to induce high-affinity SH2 binding.

The SH2-containing proteins include Src family tyrosine kinases, GAP(GTPase-activating protein), PLCγ, a subunit of phosphatidylinositol-3-kinase, tensin which is a cytoskeltal protein that links actin filaments to the membrane in adhesion plaques, and Vav which is a DNA binding protein (Fig. 2-7). It seems likely that SH2 and SH3 domains, acting in combination, allow multimeric protein complexes to form at a subcellular location appropriate to the activation of specific signal transduction pathways.

The formation of stable complexes between growth factor receptors and SH2-containing proteins such as PLC-γ1, GAP, or Src is dependent on prior growth factor binding and is generally potentiated by autophosphorylation (Margolis et al., 1989; Meisenhelder et al.,1989; Kaplan et al., 1990). SH2 and SH3 domains are the only common features of a variety of polypeptides that bind activated receptors and therefore are candidates for mediating these associations. In addition, PLC-γ and GAP belong to larger gene families, whose other members lack SH2 and SH3 domains and apparently do not interact with tyrosine kinases (Margolis et al., 1989; Meisenhelder et al.,1989; Rhee et al.,1989;

Xu et al.,1990) Direct evidence that the SH2 domains of cytoplasmic signaling proteins such as PLC-γ1, GAP, Src, and Crk are sufficient for in vitro binding to activated growth factor receptors has been obtained (Anderson et al., 1990; Moran et al.,1990).

The location of SH2-binding in the EGF receptor was shown to be its carboxy-terminal region. Full-length PLC-γ1 or a polypeptide that contains only the GAP SH2 domains binds in vitro to a carboxy-terminal EGF receptor phosphopeptide that contains four of these tyrosine phosphorylated residues but lacks the catalytic domain (Margolis et al., 1990). This is illustrated in Fig. 2-8.

2.2. Signal transduction of immunoglobulins (Igs)

2.2.1. Introduction

Besides its secreted form, membrane bound immunoglobulins (mIgs) are expressed on the surface of B cells and when stimulated with antigen, membrane Igs deliver signals for differentiation to immunoglobulin producing plasma cell. For a long time the mechanism of signal transduction of mIgM across membrane remained unclear because membrane IgM and membrane IgD are known to possess only three putative cytoplasmic amino acids Lys-Val-Lys (Hombach, 1991)(Fig. 2-9). As such a short sequence was thought to be inapropriate to elicit specific intermolecular signal transduction, some associated proteins like CD3 complex in T cells were thought to be involved in the machinery.

2.2.2. Ig-associated proteins

Until recently such Ig-associated proteins were not found when non-ionic detergent such as TritonX-100 or NP-40 were used for the solubilization of cell membrane. Recently such Ig associated proteins were found using a novel detergent digitonin which possesses weaker solubilization ability. One is a glycoprotein Ig-α with MW of 34kD which was found from mutant J558L myeloma that can express membrane IgM on its surface(Hombach et al., 1988). Its primary sequence was confirmed to coincide with the previously cloned *mb-1* gene product. Similarly, another Ig-associated protein, Ig-β, was found and shown to be identical to the product of B-cell specific transcript *B29*(Campbell et al., 1991). Igα and Igβ were shown to be necessary and enough to express five classes of membrane Igs on the plasmamembrane of non-lymphoid cells(Venkitaraman et al., 1991).

Both Ig-α and Ig-β have consensus sequence motifs containing the tyrosine found in CD3 members or IgE receptor subunits in their cytoplasmic region(Fig.2-10). These sequence are supposed to be involved in signal transduction via G protein. Recently, Grazadei et al. (1990) reported that crosslinking of mIgM on murine spleen B cell resulted in co-capping of cell-surface mIgM together with intracellular $p21^{ras}$ to the same region of cell surface. This phenomenon suggests physical and functional association of $p21^{ras}$ and B cell antigen receptor.

2.2.3. Role of tyrosine kinase in signal transduction

Tyrosine phosphorylation also was thought to be involved, rather it is thought to be the primary response in the signal transduction via mIgM. At first, increased phosphorylation of cellular components was observed within 1 min after mIgM on B cell was crosslinked with anti-IgM(Campbell et al.,1990; Gold et al.,1990; Yamanashi et al.,1991).

Second, Carter et al. (1991) reported specific inhibition of PI metabolism, intracellular rise in Ca^{2+} concentration after crosslinking of mIgM by tyrosine kinase inhibitor. They also reported a positive correlation between tyrosine phosphorylation of PLC-γ and intracellular PLC activity. Third, physical association of *src*-family tyrosine kinase *lyn* with mIgM in B cell line WEHI231 was observed(Yamanashi et al.,1991). *Lyn* was observed to be able to phosphorylate mIgM associated proteins *in vitro*, which was consistent with tyrosine phosphorylation of these proteins after mIgM crosslinking. Recently B lymphoid specific tyrosine kinase *blk* was discovered by dymecki et al. (1990). Although the association between blk and mIgM is not yet known, it is certain that it is one of activated kinases when B cell is stimulated.

The leukocyte common antigen CD45, a family of hematopoietic cell surface glycoproteins, represents a group of closely related protein-tyrosine phosphatases. As signal transduction through CD4 is amplified by specific crosslinking to CD45, a model has been proposed that CD45 activates p56*lck* by tyrosine dephosphorylation (Pingel et al., 1989; Koretzky et al., 1990). Recently, Justement et al. (1991) reported that in J558Lμm3 which is J558L expressing mIgM protein on the membrane together with α and β, introduction of CD45 restored antigen-responsive intracellular Ca^{2+} response. So also in B cell, CD45 is thought to be functionally coupled to antigen receptor complex.

2.3. Chimeric receptors

2.3.1. Introduction

Recently partial alteration of a protein by site-specific mutagenesis has been extensively tested for a lot of proteins with an aim to investigate the nature and to improve the function of target protein. Cloning of many receptor genes have made such `protein engineering' of receptor possible. Especially chimeric receptor has been noticed to be one of the tools to understand the function of receptor in molecular level. Because in most case, the MW of cell surface receptors are too large to determine the tertiary structures by authentic methods, preparation of chimeric molecules between two receptors is a powerful tool to understand their function. In this section I will introduce such examples made in the past few years.

2.3.2. Chimeric receptors of bacterial origin

Utsumi et al.(1989) reported the construction of a chimeric receptor (Taz) between aspartate binding domain of *E.coli* chemoreceptor Tar and signal generation domain of *E.coli* osmotic sensor protein EnvZ.

Tar is one of chemotaxis receptor (transducer) protein resides in inner membrane of *E.coli* or *Salmonella sp.* and known as aspartate receptor. It consists of aspartate binding domain which is in periplasmic space, two transmembrane segments one of which is at the N-terminus and the other is in the middle of the aspartate binding domain and the cytoplasmic domain, and C-terminal cytoplasmic signal generation domain. The cytoplasmic signal generation domain is known to be methylated in response to stimuli and this methyl group regulates its signal generation ability.

EnvZ is thought to be an osmosensor which regulates the expression of outer membrane porin proteins OmpC and OmpF according to alteration of osmotic pressure of medium and shows similar

topology to membrane. Its cytoplasmic domain has histidine kinase activity and one of its histidine is autophosphorylated in response to aspartate. The phosphate group is transferred to a histidine residue of OmpR which is a DNA binding protein, and the phosphorylated OmpR regulates both *ompC* and *ompF* genes alternately.

They connected these two structure genes at 3' of each transmembrane region and transformed with this plasmid *E.coli.* RU1012 which lacks EnvZ and possesses β-galactosidase reporter gene under control of *ompC* gene segment. What they discovered was that only when aspartate was added to the medium, β-galactosidase activity of transformed cell was induced (Fig. 2-11). When this plasmid was introduced into ompC$^+$,ΔenvZ strain, aspartate-dependent production of OmpC protein was observed. Participation of OmpR protein in this regulation process was proved from the fact that in *ompR*$^-$ mutant, the signal was not transduced. Thus Taz can transform aspartate binding signal to gene expression in *E.coli.* This fact is quite interesting in the view point that apparently different proteins use the same signal transduction machinery.

2.3.3. Chimeric receptor between bacterial and eukaryotic receptor

Taz is not the first attempt of making chimeras between Tar and other receptors. Moe et al. made chimeras of human insulin receptor and Tar, and reported the result twice. For the first attempt, they made a chimeric receptor IAR which encodes extracellular ligand binding domain of insulin receptor and second transmembrane and cytoplasmic domain of Tar, and expressed it in Chinese hamster ovary (CHO) cell (Ellis et al.,1986). They confirmed the expression of anticipated

chimeric protein. However, in both *in vitro* and *in vivo* conditions, insulin-dependent methylation of Tar domain was not observed.

In the next report they connected transmembrane segments and the periplasmic domain of Tar to the cytoplasmic domain of insulin receptor, designated AIR(Moe et al., 1989)(Fig. 2-11). The cytoplasmic domain of insulin receptor has, like EGF receptor, protein tyrosine kinase activity. They measured substrate kinase activity of the membrane fraction of *E.coli*. which expressed AIR with and without aspartic acid and found up to two fold increase in phosphorylation of synthetic Tyr-Glu copolymer in response to the addition of aspartate. Despite difference in substrate specificity of AIR kinase that AIR could not phosphorylate histone H2B which is a good substrate of insulin receptor kinase, the existence of at least partially common activation mechanism was suggested for both prokaryotic and eukaryotic receptors.

2.3.4. B cell-T cell chimeric antigen receptor

Goverman et al. (1990) reported chimeras between antibody and T cell receptor (Tcr). They substituted variable regions of Tcr α and β chains with that of heavy chain of hapten phosphorylcholine(PC) specific antibody (V_H). The constructed expression vectors were separately introduced into T cell line EL4 to express chimeric proteins VH-Cα or VH-Cβ. In each case chimeric protein was expressed on the membrane of EL4, and constituted a functional complex with CD3 polypeptides (Fig. 2-12). Addition of PC-conjugated Sepharose beads or anti-V_H antibody to culture medium resulted in IL-2 production after 24 hr's, which was a marker of T cell activation. This means alteration of Tcr variable domain to VH released the receptor from MHC restriction which existed

for native Tcr. In this sense, this was a direct demonstration that MHC restriction is coded by Tcr variable domain.

It was quite interesting that while VH-Cα made complex with native EL4 β, VH-Cβ made homodimer or heterodimer with EL4β. In other words, Cα was shown to be not necessary for cell-surface expression nor signal transduction of Tcr. Because Tcrα was shown to be necessary for membrane expression of native Tcrβ, perhaps the structure of Vβ made Vα necessary for its transport to membrane.

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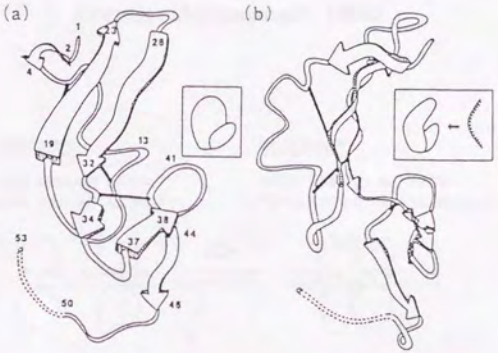
Figures

Fig. 2-1 The primary structure of EGF



(a) 正面および (b) 90度回転したもの
挿入図はEGFの立体構造を模式的に示したものである．β-シート部分を手の平，ヘアピン部分を親指とするミトンに似た構造をとる．ミトンが物をつかむようにEGFはレセプターに結合する

Fig. 2-2 The tertiary structure of EGF

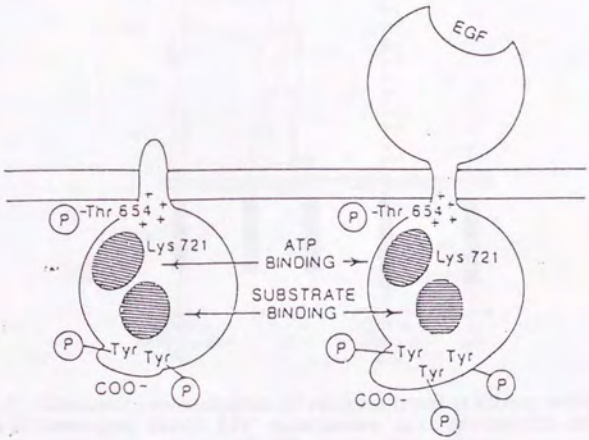


Fig. 2-3 A model for the structure of the v-erbB protein and the EGF receptor. (Schlessinger, 1986)

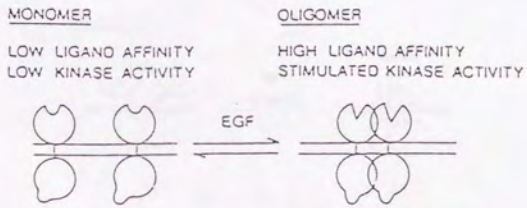


Fig. 2-4 An allosteric oligomerization model for the activation of the EGF receptor kinase by EGF. (Schlessinger, 1986)

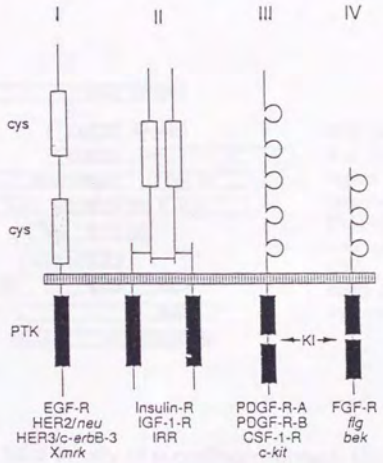


Fig. 2-5 Schematic representation of receptor tyrosine kinase subclasses (Ullrich & Schlessinger, 1990). I-IV : subclasses; cys : cystein-rich extracellular sequence; KI : kinase insert region.

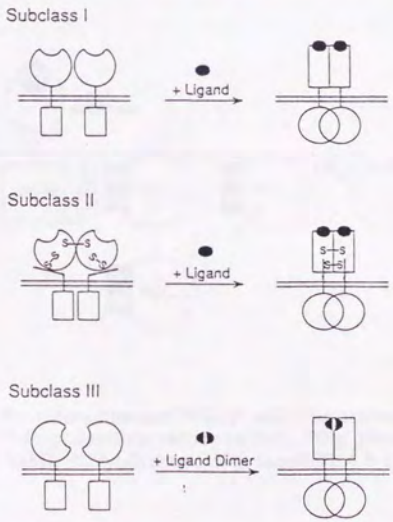
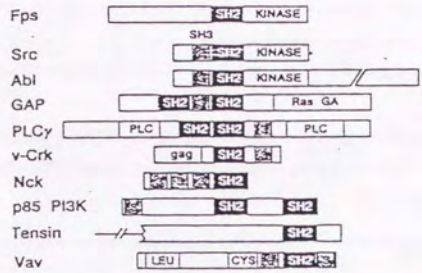


Fig. 2-6 Models of receptor subclass-specific variations of the Mechanism of activation by dimerization (Ullrich & Schlessinger, 1990) Change in shape means ligand-induced conformational change of receptor domains.



3, SH3 domain; Ras GA, Ras GTPase activating region of GAP; PLC, catalytic sequences of PLC-γ1; gag, retroviral coat protein sequence; CYS, cysteine-rich domain of Vav; LEU, leucine-rich region of Vav.

Fig. 2-7 The SH2 family of signaling proteins. (Koch et al., 1991)

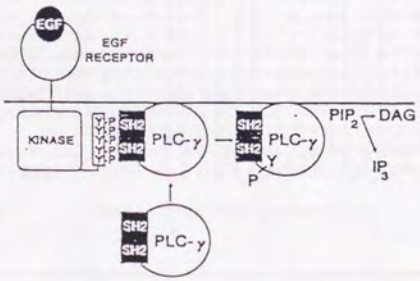


Fig. 2-8 Model for the interactions of PLC-γ1 with the activated EGF receptor (Koch et al., 1991) Y-P: phosphotyrosine residue; PIP_2: phosphatidylinositol-diphosphate; DAG: diacylglycerol; IP_3: inositol1,4,5-triphosphate

```
EGEVNAEEEGFEN LWTTASTFIVLFLLSLFYSTTVTLF KVK  Mouse   IgM
------------- ------------------------- ---  Human   IgM
----G-------- ------------------------- ---  Dog     IgM
DSSDHIWI-DN-E I--------I--F--I---AA---V ---  FISH    IgM

-QSDSYMDLEEEN --P-MC--VA----T-L--GF--FI ---  Mouse   IgD
NSDDYTTFDDVGS ----L---VA--I-T-L--GI--FI ---  Human   IgD
```

Fig. 2-9 Transmembrane sequences of membrane Igs
(reproduced from Hombach (1991))
-= identical aa.

```
h CD3-γ    GQDGVRQSRASDKQTLLPNDQLYQPLKDREDDQYSHLQGNQLRRN
mCD3-γ     -----------+-----Q-E--+-+-+----Y--+-+-+------KK
h CD3-δ    -HETG-L-G-A-T-A--R--+-V---+--R--D-A-----G--WA-N-
mCD3-δ     -HETG-P-G-AEV-A--K-E--+---+-R----T--+-S-G--WP-N-KS
mCD3-ζ     ADAYSDIGTKGERRRGKGH-G---G-STATK-T-DA-HMQT-APR
BLVgp30    LKLLRQAPHFPEISLTPKP-SD--A-LPSAPEI----SPVKPDYINLRPCP
h MB-1     RKRWQNEKLGL-AGDEYEDEN--EG-NLDDCSM-EDISRGLQGTYQDVGSLNIAD
mMB-1      RKRWQNEKFGV-MPDDYEDEN--EG-NLDDCSM-EDISRGLQGTYQDVGNLHIGD
mB29             DKD-GKAGMEE-HT-EG-NIDQTAT-EDIVTLRTGEVKWSVGEHPGQ
rFcεRI-γ     RLKI-V-KA-IASREKS-AV-TG-NT-NQET-ET-KHEKPPQ
rFcεRI-β       Y-IGQEFE.RSKV-D-R--EE-.HVYSPI--A-EDTREASAPVVS

    Consensus:      DxxxxxxxDxxYxxLxxxxxxxYxxL
                    E       E                I
```

Fig. 2-10 Consensus sequence motif of cytoplasmic region of receptor-associated transmembrane proteins (Hombach (1991)). All the sequences except CD3ζ and BLVgp30 begin with first cytoplasmic residue. All the sequences except mb-1 and B29 end with C-terminus of proteins.
h= human; m= mouse; r= rat; .= lacking; -= identical aa.

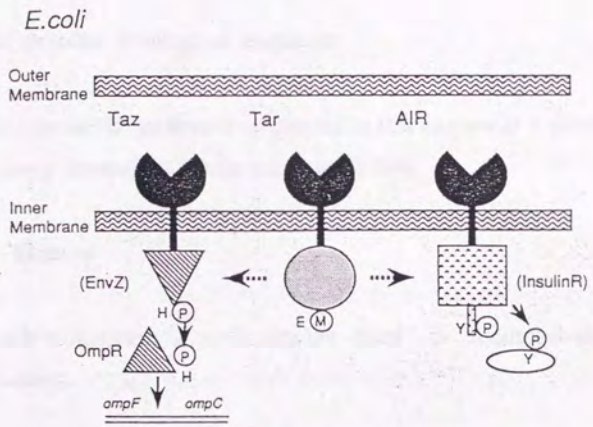


Fig. 2-11 Schematic drawing of bacterial chimeric receptors. Large gray circle in Tar means cytoplasmic domain of Tar which accepts methyl group at glutamate residues.

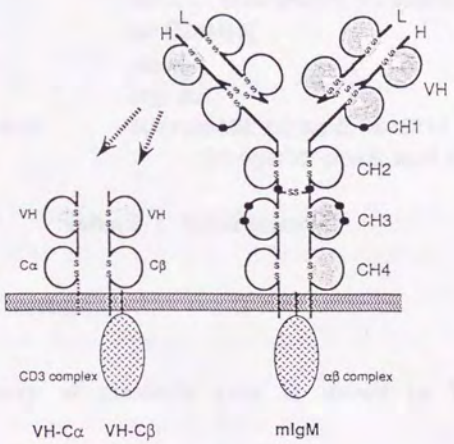


Fig. 2-12 Schematic drawing of Ig-Tcr chimeric receptors.

Chapter 3. Materials and methods

3.1. Molecular biological methods

Almost all the protocols employed in this chapter is a modification of laboratory manual by Sambrook et al. (1989).

3.1.1. Strains

Followings are *E.coli* strains used for recombinant DNA manipulations.

HB101	*supE*44, *hsdS*20($r_B^-m_B^-$), *recA*13, *ara*-14, *proA*2, *lacY*1, *galK*2, *rpsL*20, *xyl*-5, *mtl*-1
JM109	*recA*1, *endA*1, *gyrA*96, *thi*, *hsdR*17, *supE*44, *relA*1, λ⁻, Δ(*lac-proAB*), [F', *traD*36, *proAB*⁺, *lac* I^qZΔM15]
GM33	*dam*3
BW313	*ung*, *dut*
BMH71-18 *mutS*	Δ(*lac-pro*AB), *thi*, *supE*, *mutS*215 ::Tn10(tet^r)/F'*proAB*, *lac*I^q ZΔM15

Table 3-1 *E.coli* strains

3.1.2. Plasmid vectors

The summary of plasmids used is shown in Table 3-2.

pSV-Vμ1	Neuberger, S.(1983)
pE7	Merlino, G. T. (1985) (From J.C.R.B.-DNA)
pHER	Ueda, H. (*using fragment cloned by Yagi, S.)
pAE7.7	Yamamoto, T.(1983)
pABμ-11	Bothwell, A. (1981)
pUC119	Vieira, J., & Messing, J. (1987).

Table 3-2 Used plasmid vectors

Human EGFR cDNA was subcloned into Eco RI-SmaI sites of pUC19 from cDNA sequence of Apa I 2141 - Sca I 3968, which was cloned from human placental library by using pE7 as a probe, using synthetic EcoRI-BglII-ApaI adapter (5'-AATTCAGATCTCCACTGG-GATGGTGGGGCC-3' and 5'-CCCACCATCCCAGTGGAGATCTG-3') and designated pHER. Nucleotide sequence was confirmed to coinside with published sequence. Plasmid pE7 was supplied by Japan Cancer Research Bank.

3.1.3. Restriction endonuclease digetion and gel electrophoresis

Plasmid DNA was digested by restriction endonuclease(s) in a 50μl reaction mixture in 1.5ml sterilized microtube in which several μg of DNA and 5μl 10x appropriate digestion buffer was contained, and digested for 1~2 hr at suitable temperature (usually 37°C). Completeness of digestion was confirmed by analysis of one tenth of reaction mixture by agarose gel electrophoresis using 0.6% agarose (Nippon Gene) and 1xTAE. Usually, the remaining reaction mixture was mixed with 70μl 49:49:2 phenol/chloroform/isoamylalchol equilibrated with TE, vortexed,

and centrifuged at 12krpm 3 minutes. Upper phase was transferred to a new microtube, mixed with 5µl 3M sodium acetate (pH5.2) and 100µl ethanol, and stored at -80°C for 15 minutes. This was centrifuged at 15krpm 5 minutes 4°C, and supernatant was removed. 160µl 70% ethanol was used for rinse of pellet and after brief centrifugation, supernatant was completely removed and the pellet was dried under vacuum. Pellet was dissolved in ~50µl TE containing 0.025% BPB and 4%(W/V) sucrose and applied to agarose gel electrophoresis.

3.1.4. Purification of fragments and ligation

Purification of DNA fragments from agarose gel was done with Geneclean™ kit(Bio101) or with DNA-prep kit(Dia-ayatron, Asahi Glass). Briefly, the DNA containing gel was dissolved in 3 vol. NaI solution at 55°C 5 minutes. This was mixed with ~5µl glassmilk suspension and stored at 0°C 5 minutes, centrifuged, and the pellet was washed 3 times with NEW wash solution containing NaCl, ethanol and water. Then the adsorbed DNA was eluted from pellet in 5~10µl TE during twice incubations at 55°C 3 minutes and centrifugations.

The ligation was accomplished with DNA ligation kit (Takara) according to the manufacturer's instructions. Usually, TE solution containing DNA mixture up to 2µl were mixed with 8µl A solution containing probably PEG and NaCl and 1µl B solution containing T4 DNA ligase and ATP. This was incubated at 16°C for 15 to 60 minutes.

3.1.5. Transformation and preparation of plasmids

Preparation of competent cell and transformation of DNA was done according to Hanahan et al. (1983). In most case, transformation

efficiency of 10^6-10^8/μgDNA was obtained for HB101 or JM109. Usually, 5μl of ligation mixture and 50μl frozen stock of competent cells were mixed and kept on ice for 30 minutes, applied with heat shock at 42°C 30 seconds, mixed with 200μl SOC medium, and incubated at 37°C for 30-60 minutes. Plating of 100-250μl on an antibiotics-containing LB-agar plate usually gave sufficient counts of colonies after overnight incubation at 37°C. Colonies were picked up, transferred to antibiotics-containing 5ml LB culture tubes and shaken 8~16hr at 37°C. 1.5ml to 3ml saturated culture was used for rapid preparation of plasmids by alkaline lysis method. Usually one tenth of RNase treated DNA solution was used for restriction enzyme analysis.

3.1.6. Site directed mutagenesis

Site directed mutagenesis was done according to Kunkel et al. (1987). Briefly, the DNA fragment to be mutated was subcloned into pUC119. The plasmid was transformed to BW313 and transformants were infected with M13KO7 helper phage at m.o.i. = 2~10. After shaking for 16 hrs at 37°C, the culture supernatant was recovered and single-stranded, uracil-containing phagemid was collected by PEG precipitation and phenol extraction. The DNA and mutagenic primer was annealed, polymerized to ccc dsDNA by T4 DNA polymerase and T4 DNA ligase, and transformed to BMH71-81 mutS. About 60% of transformants were mutants, which was confirmed by nucleotide sequencing using modified T7 DNA polymerase (Sequenase™, USB).

3.2. Animal cell handling

To express chimeric receptor protein, a mammalian cell line J558L was used because this myeloma cell has a merit that it expresses λ1 light chain.

3.2.1. Cells

The cell lines used are following.

J558L	mouse plasmacytoma	Oi, V. T.
A431	human epidermoid carcinoma	Giard,D.J. & Aaronson,S.A.

Table 3-3 Cells used for expression

J558L was a kind gift from Masaru Taniguchi in Chiba University. A431 epidermoid carcinoma was supplied by Japan Cancer Research Bank.

3.2.2. Cell culture

J558L cell was cultured in 95% Dulbecco Modified Eagle medium (DMEM) (Nissui pharmacy), 5% FCS, 10mM Hepes pH7.4, 10μg/ml kanamycin. Cells were cultured in 37°C 5%CO_2 in humidified atmosphere. Storage of frozen cells were done in 90% FCS and 10% DMSO at -80°C or in liquid N_2. A431 was cultured almost the same condition but in 90%DMEM + 10% FCS, and passaged by trypsinization in 0.25% trypsin and 0.2% EDTA.

3.2.3. Transfection, selection and cloning of transfectants

J558L cells were transfected with gel-filtrated plasmids by electroporation with hand-made apparatus. 20μg plasmid DNA and 10^7 cells were mixed in 0.5ml Hanks' buffer(Nissui pharmacy) and 8-16 pulses of 1kV/cm,200μsec were applied to a plastic chamber with 4mm distance. Transfectant was selected in DMEM plus 10% FCS, 6μg/ml mycophenolic acid and 250μg/ml xanthine from 24hr after transfection. About 2 weeks later, colonies were cloned by limiting dilution in 96 well plates and the clones with relatively high protein expression are screened by ELISA of cell lysate.

3.2.4. Flow cytometric analysis

For staining of membrane, $2x10^6$ cells were collected, washed once in 1ml FACS medium (Hanks' buffer+2% BSA, 0.2%NaN_3), stained with 10μg/ml FITC-anti mouse IgM (Tago) in 50μl FACS medium on ice for 30 min., washed twice in 700μl FACS medium and resuspended in 1ml FACS medium. All the centrifugation was 1krpm 5min 4°C in microfuge. Hanks' buffer was often substituted with PBS. To enhance the sensitivity, multifold staining was done using rabbit anti-mouse IgM (Zymed), biotinated anti-rabbit Ig (Amersham), and FITC-avidin. FITC-avidin was made from Avidin(Sigma) and FITC(Wako).

Flow cytometric analysis and cell sorting was done with either EPICS 753 or EPICS-C(CS) flow cytometer(Coulter) according to manufacturer's instructions.

3.2.5 Subcellular fractionation

Subcellular fractionation of intact or transfected J558L cells was done according to Bole et al.(1986) with some modification. $5x10^7$ cells

were harvested by centrifugation at 1000g for 5 min at 4°C, and first washed twice with PBS and then twice with 0.25M sucrose in 5mM Hepes buffered to pH6.8. The cells were then resuspended in 2ml of isotonic sucrose and homogenized in a tight-fitting dounce homogenizer. Nuclei and cellular debris were removed by centrifugation at 800g for 10 min. The postnuclear supernate was then layered on top of a discontinuous sucrose gradient containing 1ml/2.0M, 3.4ml/1.3M, 3.4ml/1.0M, and 2.75ml/0.6M sucrose in 5mM Hepes (pH6.8) in a Beckman 14x89 mm Ultra-Clear tube. After 2h of centrifugation at 40,000 rpm in a Beckman SW 41 rotor, ~19 fractions containing 10 drops each were collected from the bottom of the tubes. Each fraction was then assayed for endoplasmic reticulum and Golgi marker enzymes.

NADPH-cytochrome C reductase was used for marker enzyme for endoplasmic reticulum. Measurement was done according to Kasper (1971).

Galactosyltransferase activity was measured for Golgi apparatus. Membrane fractions were incubated in a final volume of 40µl containing $5x10^4$ cpm UDP-Galactose [Galactose-^{14}C(U)] 337.0mCi/mmol, 0.28mg ovalbumin (Sigma), 50mM Tris, pH7.5, 20mM $MnCl_2$, 0.5% Triton X-100. Reactions were incubated at 37°C for 60 min. 30µl of each assay was then spotted on Whatmann 3-MM filter paper and dried at room temperature. The filter paper was then washed twice in 10% trichloroacetic acid, 2% phosphotungstic acid, and 1% pyrophosphate at 0°C for 15 min. The filter paper was then washed in methanol, dried, and counted by liquid scintillation counter (Packard).

3.3. Biochemical analysis of proteins

Characterization and analysis of expressed protein was done with authentic biochemical methods.

3.3.1. Metabolic labeling and immunoprecipitation

The clones were metabolically labeled with 100μCi of [^{35}S]methionine for 4 hrs to overnight in 4ml methionine-free MEM (Nissui Pharmacy) +1% FCS, washed twice in PBS, lysed in lysis buffer (20mM Hepes pH7.5, 150mM NaCl, 10% glycerol, 1% TritonX-100, 1.5mM $MgCl_2$, 1.0mM EGTA, 1mM PMSF, 10μg/ml aprotinin, 10μg/ml leupeptin). Samples were centrifuged at 15krpm, 10 min 4°C, then immunoprecipitated for 60min at 4°C with antibodies prebound to protein A-Sepharose beads (Sigma) for 30min at 4°C. Beads were washed 4 times with 50mM Hepes pH8.0, 500mM NaCl, 0.1% SDS, 0.2% TritonX-100, 5mM EGTA and once with the same buffer with 150mM NaCl.

3.3.2. Measurement of autophosphorylation activity

Purification of unlabeled lysate by NP-cap-Sepharose was done batchwise for 60 min at 4°C on a rotating wheel, washed 4 times with $HNTG_{500}$ (20mM Hepes pH7.5, 500mM NaCl, 0.1% TritonX-100, 10% glycerol), once with HNTG (Same as $HNTG_{500}$ except that NaCl concentration is 150mM) and eluted with 1mM NP-caproate in HNTG. NP-cap-Sepharose was made by conjugating EAH-Sepharose-4B (Pharmacia) with NP-caproate-ONS at 20°C 16 hrs. Then 20μl of eluate was mixed with 10μl of reaction mixture (15mM $MnCl_2$,200μM Na_3VO_4,50nM ~3μCi [γ^{32}P]ATP) and incubated for 10 min at 37°C.

NP-caproate-ONS ester and NP-BSA was made from NP-caproate (Cambridge Research Biochemicals) as described(Pohlit et al., 1966).

NP/BSA ratio was determined to be 3~4 by measurement of A_{430} and BSA concentration by Lowry method.

3.3.3. SDS-polyacrylamide gel electrophoresis and autoradiography

Samples were boiled in reducing sample buffer (3% SDS; 50mM Tris,pH6.8; 10% glycerol; 0.05mg/ml BPB; 1% 2ME) or in unreducing sample buffer (-2ME). Gel electrophoresis was done according to the method of Laemmli (1970). For autoradiography, the gel was dried on gel drier (ATTO) and exposed over Fuji RX X-ray film overnight. For fluorography, the gel was fixed in 30% methanol-10% acetic acid for 20 min, then soaked in EnLightning (Du pont) for 20 min, dried, and exposed over Fuji RX X-ray film overnight at -80°C.

3.3.4. Enzyme-linked immuno adsorbent assay

Enzyme-linked immuno adsorbent assay (ELISA) was frequently used for quantitation of antibody or its chimeras in supernatant or within cell. When intracellular protein was analyzed, up to 10^6 cells were lysed in 100 μl lysis buffer containing 1% TritonX-100, 1mM PMSF in phosphate buffered saline (PBS), centrifuged for 5min at 15krpm 4°C to remove nucleus, and used as a sample. Sequentially diluted samples were applied to 96 well microtiter plate which was precoated with antigen NP-BSA (1mg/ml) for 1 hr and blocked with 1% BSA in PBS for additional 1 hr. After 2hr at r.t. samples were removed and wells were washed with 0.1% Tween-20 in PBS for 4 times. Incubation with HRP-conjugated goat anti-mouse IgM (Tago) diluted to 1/1000 in PBS was continued for 1hr followed by removal and 5 times of extensive washing.

Chlorimetric measurement of HRP activity was accomplished by published procedure.

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Chapter 4. Cell surface expression of chimeric IgM-EGF receptor protein in myeloma cell

4.1. Introduction

Chimeric protein has usually several unnatural features such as relative unstability compared with natural protein. To make use of chimeric proteins, a system for stable expression of the proteins must be explored and established. The protein expression systems are divided into *in vitro* and *in vivo* systems. *In vivo* systems are divided into prokaryotic (bacterial) and eukaryotic (yeast, animal cell, plant cells etc.) systems. I used animal cell expression system because native EGF receptor was expressed on the membrane of animal cells. Because I have to express antibody composed of heavy and light chain, a cell line which expresses light chain was convenient because I do not have to make an expression vector for them. So I decided to use murine myeloma J558L which produces and secretes λ1 light chain. However, during my experiment a problem arose that it was reported that gene-introduced myeloma J558L could not transport membrane IgM protein to plasmamembrane (Sitia et al., 1987). I was very annoyed by this report but on the contrary the chimeric receptor was found to be efficiently transported to plasmamembrane.

4.2. Construction of the expression vectors for chimeric receptor

The schematic primary structures of the chimeric receptors I made (heavy chains) are shown in Fig.4-1A. One has EGF receptor transmembrane and cytoplasmic region and the other has *erbB*

cytoplasmic sequence which is an avian homologue (oncogene) of EGF receptor connected to human EGF receptor transmembrane sequence. Both constructs have the antigen NP (4-hydroxy-3-Nitrophenyl acetic acid, Fig. 4-1B) -specific variable region sequence with its promoter together with genomic IgM sequence which contains Ig enhancer. Polyadenylation signal derived from SV40, *Ecogpt* selection marker under the control of SV40 early promoter, and a part of pBR322 sequence which enables duplication of the plasmid in *E. coli.* is also encoded in these vectors. The protocols to construct the expression vectors are illustrated in Fig. 4-2A~B.

4.3. Confirmation of expression of the receptor proteins

Constructed expression vectors were purified by gel-filtration through agarose column to remove RNA and other small compounds which may be toxic to eukaryotic cell. The purified plasmids were introduced into myeloma J558L by electroporation. Cells were cultured in the presence of mycophenolic acid for 14 days to select gene-introduced cells. On the other hand, transient expression of the EGFR-specific mRNA 48hrs after transfection was detected by Northern blot analysis (Fig.4-3). While a clear expression of the EGFR-specific mRNA was detected in pSV-VμER transfectants, pSV-Vμ1 transfectant and non-transfected J558L showed no expression of the RNA.

After selection, the colonies were collected, cultured without selection, purified from dead cells, and confirmed for their expression of IgM protein. When ELISA of the cell lysate was done (Fig.4-4), the increase of expression of IgM that was able to bind NP-BSA was found in pSV-VμER and pSV-Vμ1 transfectants. Then the cells were cloned and tested for their expression of IgM by ELISA. After several positive

clones were obtained, one of them was labeled with [^{35}S]-methionine overnight, harvested, lysed, immunoprecipitated with rabbit anti-mouse IgM, and detected by SDS/7.5% PAGE followed by fluorography (Fig.4-5). This indicates immunoprecipitation of 120-140 kD in transfectants, which corresponds well to predicted MW of chimeric receptors. The amounts of expressed receptors were almost the same.

4.4. Assay of protein tyrosine kinase activity

The immunopurified receptor was used for the subsequent assay of autophosphorylation activity. The autoradiography of SDS/7.5% PAGE after kinase reaction containing [γ^{32}P]ATP is shown in Fig.4-6. The same pattern as seen in Fig. 4-5 was observed and the intrinsic autophosphorylation activity of anti-IgM purified chimeric receptors was confirmed. The activity of VμerbB was observed to be almost the same as that of VμER.

4.5. Quaternary form of chimeric receptors

The autophosphorylation activity was also observed for antigen NP-affinity purified receptor protein. The cell lysate of the transfectant was mixed with NP-caproate-Sepharose column, washed, and bound receptor was eluted with 1mM NP-caproate. The kinase activity of eluate was measured by autoradiography of SDS-PAGE after kinase reaction including [γ^{32}P]ATP. Inclusion of exogenous substrate poly Glu-Tyr into reaction mixture containing affinity-purified VμER protein resulted in phosphorylation of substrate(Fig. 4-7A). As shown, phosphorylated substrate with broad MW range together with autophosphorylated

receptor was observed for lane VμER-10 and not for J558L. This result indicates that the kinase of the chimera was tyrosine kinase.

PAGE analysis of autophosphorylated receptor under unreducing condition (Fig.4-7B) indicates that MW of unreduced receptor was about 350 kD which is more than twice the size of that in reduced condition. Because the chimera has antigen binding ability, it should be composed of heavy(H) and light(L) chains to constitute antigen binding site(s). The MW of H and L chain were ~140kd and 30kd as shown in Fig.4-5, so the quaternary form of VμER seemed to be H_2L_2 heterotetramer which could be predicted from native mIgM structure.

4.6. Confirmation of cell surface expression of chimeric receptor

In myeloma J558L, mIgM was reported to be retained in RER and not expressed on plasmamembrane (Sitia et al., 1987). To determine whether the obtained transfectant expressed VμER protein on plasmamembrane or not, fluorescence labeling of cell surface IgM was done with rabbit anti-mouse IgM, biotinated donkey anti-rabbit Ig and FITC-labeled avidin. Because clearly positive cells are not observed by fluorescence microscopy, flow cytometric analysis of labeled cells were done(Fig.4-8). According to the result, pSV-VμER and pSV-Vμerb transfectants showed increased fluorescence which indicated cell surface expression of these proteins with orientation of IgM to outside. On the contrary, pSV-VμmM transfectant which expressed W.T. mIgM showed no obvious fluorescence increase (not shown; see chapter 5).

4.7. Subcellular fractionation and distribution of chimeric receptors

To confirm the transport of VμER protein from another point of view, subcellular fractionation of VμER transfectant was done by stepwise sucrose density gradient method. The distribution of VμER or other proteins in each organelle were determined by ELISA and marker enzyme assays. The result is shown in Fig.4-9. The peak for RER, Golgi apparatus, and SER was seen for VμER protein. On the contrary, for mIgM no peak was observed except that for RER. This pattern corresponds well to the previous report (Sitia et al.,1987). From this experiment we can conclude that VμER protein was at least transported to Golgi apparatus. The facts that rate limiting step of protein secretion is transport from RER to Golgi (Lodish, 1988) and that the plasma-membrane fraction corresponds to SER fraction (Sitia et al., 1987) will lead us easily to guess that it is also transported to plasmamembrane.

4.8. Discussion

We have shown here the efficient membrane expression of chimeric IgM-EGFR molecules in myeloma J558L which normally denies membrane expression of membrane IgM or membrane IgD (Sitia et al.,1987; Hombach et al., 1988). This was directly demonstrated for the chimera with the EGF receptor transmembrane sequence by analysis of intracellular localization of the synthesized molecule. For the chimera with the mIgM transmembrane sequence we have also shown the elevated cell surface IgM-associated fluorescence with flow cytometry.

Myeloma is widely used for the production of monoclonal antibody as a fusion partner of immunized B cell or the host for the expression of genetically modified antibodies such as humanized chimeric antibody or antibody-tagged enzymes (Neuberger et al.,1984; Neuberger, 1985).

However, while myeloma secretes these molecules quite efficiently, it was also known that myeloma did not expressed membrane Igs nor other cell surface antigens unlike its precursor B cell (Sitia et al.,1987). There was a conjecture that myeloma was not capable of expressing cell surface receptor efficiently. Our result showed this was not correct. Similar observation had been reported by Hombach et al. (1988) who succeeded to get membrane IgM positive μ-gene transfected J558L mutant. However direct demonstration that wild type J558L could express membrane IgM with altered transmembrane domain did not appear before our result (Hombach et al.,1990).

Myeloma cell has several advantages as a host to produce bioactive materials in practical culture that i) it has shorter doubling time compared to other cell lines, ii) the passage is easy because simple dilution is enough, iii) the maximum cell density is relatively high ($>10^6$ cells/ml) in the absence of carrier and iv) suitable serum free media was already developed with relatively reasonable price. These features are considered to be important to produce large amount of proteins with higher cost performance.

Chimeric antibodies combined with other enzymatic domains have been reported by several researchers (Neuberger et al.,1984; Williams & Neuberger,1986; Schnee et al., 1987; Casadei et al., 1990). All of these were reported to have both antigen specificity and enzymatic activity which enables easy purification or selective targeting of the enzyme. In the latter case, tissue-type plasminogen activator was combined to anti-plasmin antibody which enables selective targeting of this hybrid molecule to blood coagulation. In our case, purification of chimera by NP-Sepharose was possible and active kinase of both human EGF receptor and avian erythroblastosis virus (AEV-H) *erbB* gene product were easily purified. Thus purification of transmembrane protein in the

presence of detergent was demonstrated to be equally easy compared to the previous case in the absence of detergent.

Although transmembrane signaling was shown elusive, the obtained molecule has advantage in offering novel purification means in the purification of tyrosine kinase, which will lead to crystallization of tyrosine kinase domain which no one has ever succeeded.

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Figures

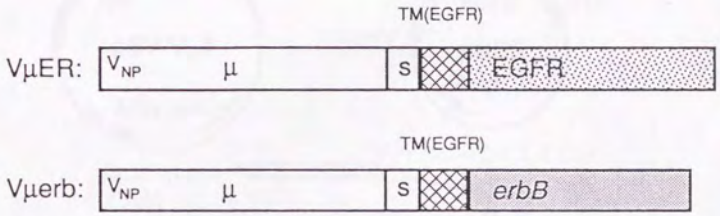


Fig. 4-1A Structure of hybrid receptors (Heavy chain). V_{NP}: hapten NP-specific variable region derived from hybridoma B1-8; μ: IgM constant region; s: secreted tailpiece; TM: transmembrane sequence

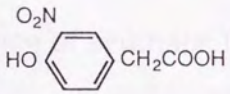
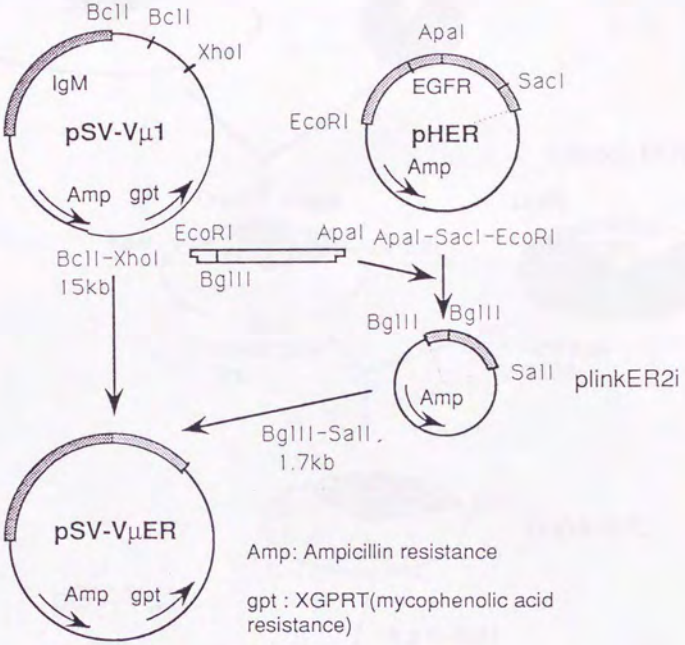


Fig. 4-1B Structure of NP



Sequence of synthetic linker

AATTCAGATCTCCACTGGGATGGTGGGGGCC
GTCTAGAGGTGACCCTACCACCC

Fig. 4-2A Construction of pSV-VμER

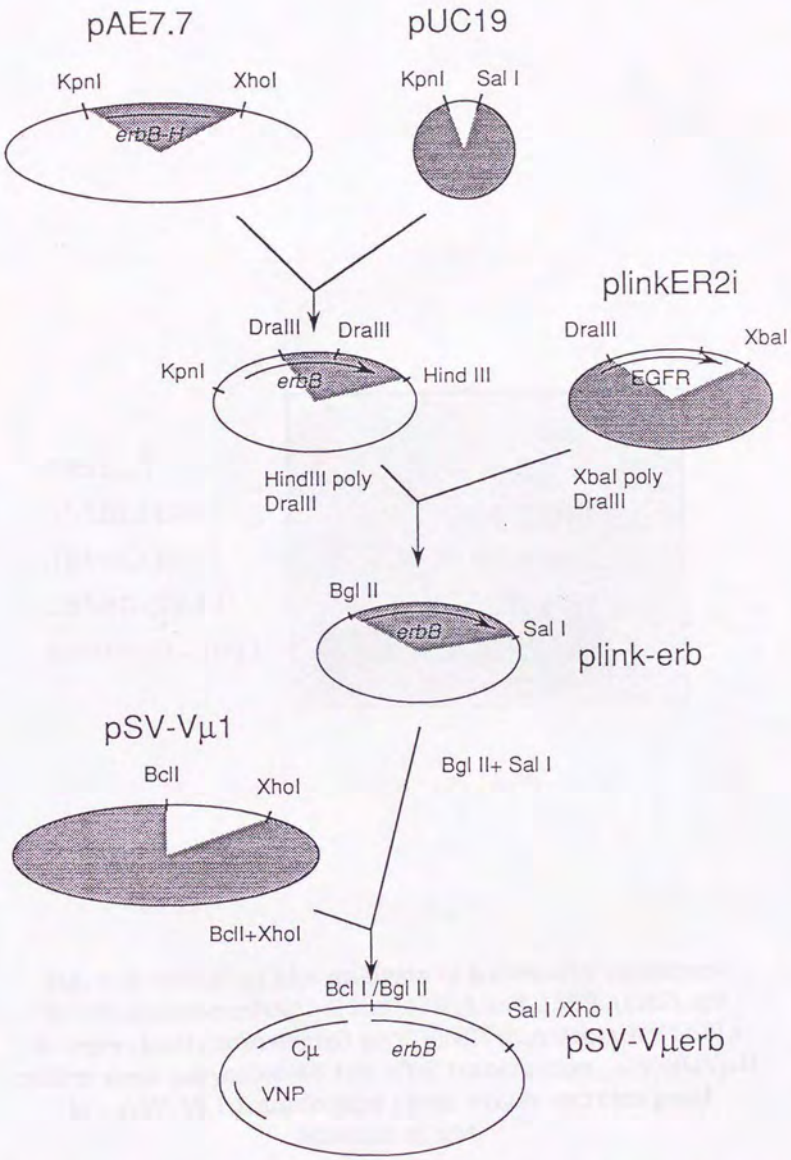


Fig. 4-2B Construction of pSV-Vμerb. Hatched region is used for the next construct.

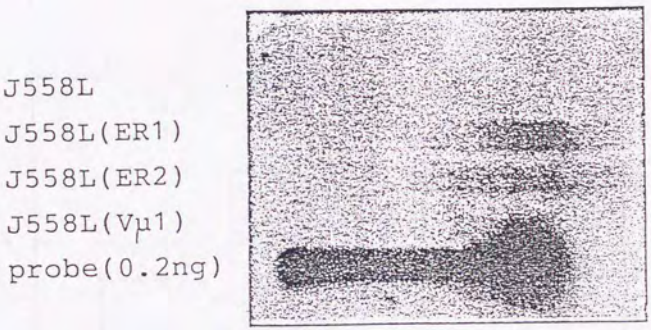


Fig. 4-3 Northern blot analysis of transiently expressed EGFR-specific mRNA. J558L(ER1) and J558L(ER2) are independently transfected pSV-VμER transfectant J558Ls which were harvested 48 hrs after transfection. J558L(Vμ1) is a pSV-Vμ1 transfectant clone which secretes good amount of IgM.

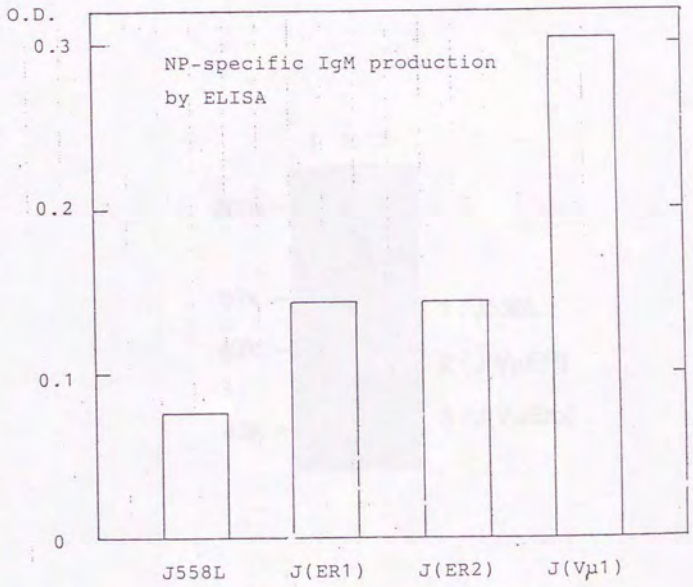


Fig. 4-4 NP-specific IgM production of stable transfectants before cloning. Cells ($1x10^6$) used are the same as in Fig. 4-3. $O.D._{420}$ shows relative amount of NP-specific IgM detected by ELISA using alkaline-phosphatase conjugated Abs.

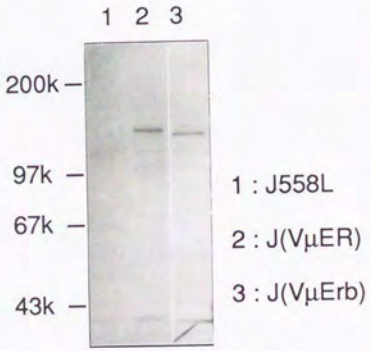


Fig. 4-5 Immunoprecipitation of hybrid receptors. [^{35}S]-labeled cell lysate was immunoprecipitated with αIgM, separated by SDS/7.5% PAGE, and detected by fluorography.

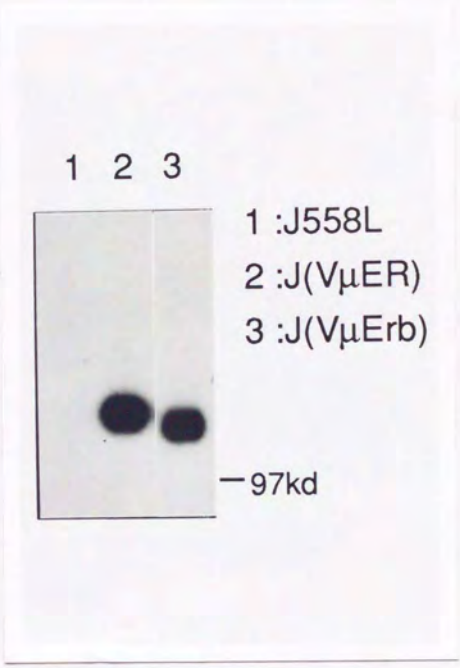


Fig. 4-6 Autophosphorylation of hybrid receptors. Immunoprecipitated receptor was incubated with [γ^{32}P]-ATP, separated by SDS/7.5% PAGE, and detected by fluorography.

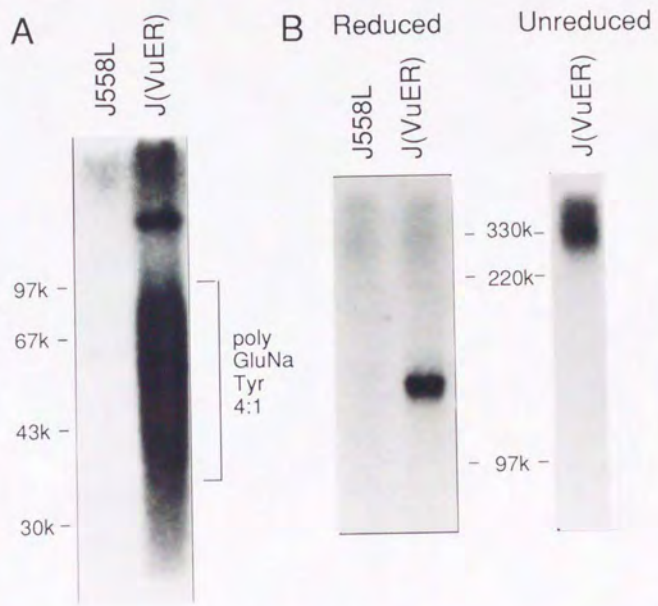


Fig. 4-7 Characterization of NP-affinity-purified receptor. A: Substrate phosphorylation activity detected by fluorography after reduced SDS-PAGE; B: Autophosphorylated receptor detected by fluorography after reduced or unreduced SDS-PAGE.

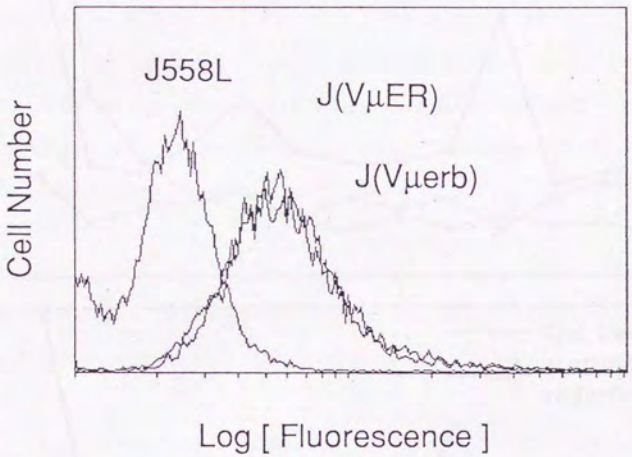


Fig. 4-8 Relative expression of IgM molecule on the cell surface of transfectant clones detected by flow cytometry.

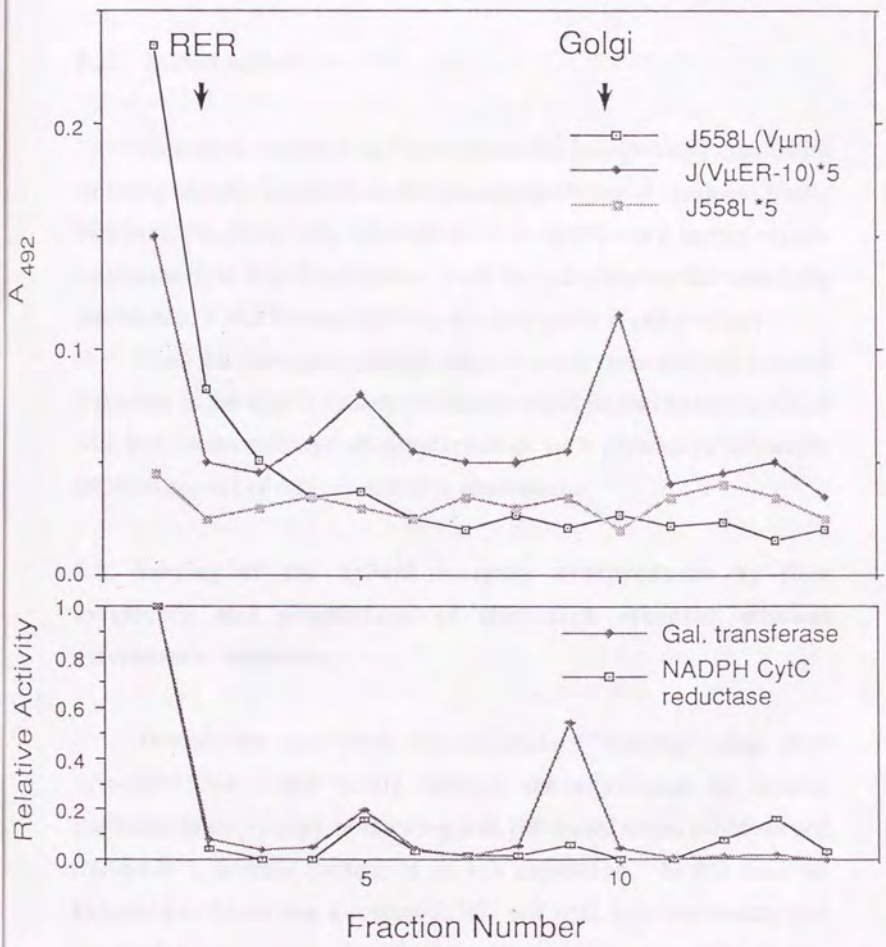


Fig. 4-9 Subcellular distribution of IgM in transfected J558L cells. Relative amount of NP-specific IgM was detected by ELISA using peroxidase conjugated Abs. Marker enzyme assay was done using an independent sample.

Chapter 5. Identification of transport-inhibitory sequence of membrane IgM in myeloma cells

5.1. Introduction

As seen in chapter 4, we have succeeded in expressing IgM-EGFR chimeric receptor molecule on the plasma membrane of myeloma J558L. However, the reason why we could do so in J558L which cannot express membrane IgM is still unknown. So if we can discover the underlying mechanism, it will be valuable from the view point of cell biology.

From the view point of application, it is still more valuable because it teaches us the way to control membrane-bound protein transport, which will lead to the efficient overproduction of such proteins or ultimately artificial control of cell-cell adhesion phenomena.

5.2. Sorting of the hybrid receptor overproducer by flow cytometry and acquisition of truncated receptor without cytoplasmic sequence.

Though we confirmed the cell-surface receptor using flow cytometry, we could hardly confirm the expression by normal fluorescence microscopy or rosetting with NP-coated sheep red blood cell (NP-SRBC), perhaps because of its low expression. At that time we happened to know that a mutant J558L cell with high membrane IgM expression was obtained with successive flow cytometric sorting (Hombach et al., 1988). So we decided to sort our cells to obtain highly expressive cells.

About 5% of positive cells, which have been stained with biotinated anti-murine Ig plus FITC-labeled avidin, were sorted using flow

cytometric cell sorter EPICS753 (Coulter). The sorted cells were cultured for 2 weeks, stained again, and analyzed by fluorescence microscopy and flow cytometry.

As a result, an increased population of the cells with remarkable elevation of fluorescence intensity was obtained (Fig. 5-1). So we cloned the cells and obtained several clones with both low and high expressions of the receptor on membrane. The flow cytometric analysis of the representative clones are shown (Fig.5-2).

Then the autophosphorylation activity of the receptors on these clones were measured (Fig.5-3). While we could observe relatively strong activity with clone J(VμER)-10 which express small amounts of receptor, we could scarcely observe the activity from J(VμER)-6,-7, or -9 which had many cell-surface receptor molecules. To reveal the reason, we measured the amount and the length of receptor-specific RNA by Northern blotting(Fig 5-4A) and the amount and the weight of the protein by immunoprecipitation(Fig.5-4B). With Northern blotting probed with IgM constant region, overproduction of J(VμER)-6 IgM RNA was confirmed. However, the immunoprecipitation with anti-murine IgM revealed strikingly small molecular weight (~80kd) of the receptor protein of this clone, which was 60kd smaller than expected molecular weight. Because this difference corresponded well to the MW of the cytoplasmic region of EGFR, truncated receptors without cytoplasmic domain was thought to be overproduced in these cell lines with unknown reason .

5.3. Comparison of transport efficiency of intact and truncated receptors

Why could truncated receptor molecules be expressed on plasma membrane so efficiently? To answer this question, a comparison between the amount of intact and truncated receptors in the cell was done. J(Vμerb), J(VμER)-10 and J(VμER)-6 cells were metabolically labeled with [^{35}S]-methionine, lysed and the equal counts of lysate was used for immunoprecipitation analysis. As shown in Fig.5-5, 16 fold amount of receptor protein in weight was expressed in J(VμER)-6 compared with J(VμER)-10. It means about 32 fold molar receptor was expressed. From the data presented in Fig. 5-2, we calculated the ratio of the number of cell surface receptors of two cell lines: the ratio of log-integral of the two histograms will provide the ratio of cell surface receptors. In this case the ratio was 21.7 (VμER-6 vs. VμER-10) and 23.8(Vμerb vs. VμER-10). As these ratios correspond well to the ratio measured by immunoprecipitation, we can conclude that the transport efficiency of both intact and truncated receptors seem to be almost the same.

5.4. Efficient cell-surface expression of the "ready-made" truncated receptor

If overproduced receptor lacks its cytoplasmic region, there is a possibility that the receptor with no cytoplasmic region can be highly expressed on membrane: if the construct with no cytoplasmic region is made, the expressed protein should be expressed in large amount and efficiently transported to membrane. Based on such hypothesis, I made a new expression vector pSV-VμsE which would express hybrid receptor without whole cytoplasmic region (denote VμsE)(Fig.5-6).

The plasmid was introduced into J558L by electroporation and selected as before. After staining with fluorescence-labeled anti-Ig, most

of the selected cells showed bright fluorescence(not shown). This provided a clear evidence that J558L was capable of expressing intact receptor molecules on its plasma membrane when it has adequate transmembrane/ cytoplasmic sequences.

5.5. Identification of transport-inhibitory sequence in membrane-IgM using chimeric protein

VμsE protein had the same primary structures as the membrane IgM(VμmM) except C-terminal small region containing transmembrane sequence. So the remarkable difference in transport efficiency must be explained by the difference in C-terminal residues. To explore the precise structural requirements for the retention of membrane IgM in ER, I planned several experiments.

At first, I made a chimera of VμsE and VμmM to locate the retention-responsible sequence. The predicted C-terminal amino acid sequences to be expressed by the constructs are shown in Fig.5-7. Although a reciprocal chimeric construct pSV-VμmE was made, but it expressed not the expected molecule but the secreted-type IgM which seemed to be translated from alternatively-spliced mRNA(data not shown).

These individual vectors were simultaneously introduced into J558L, cultured in selection medium, and analyzed for cell surface IgM expression. The result of flow cytometric analysis is shown in Fig.5-8. The profile of VμsM-introduced cell shows intermediate pattern of VμsE and VμmM transfectants. Then we cloned each transfectants and analyzed representative transfectants(Fig.5-9). A similar tendency was observed.

Next, these clones were fractionated to determine the subcellular localization of the expressed molecules. Cells were homogenized and

fractionated after centrifugation on sucrose stepwise density gradients(Fig.5-10). In the profiles for VμsE and VμsM, a peak was observed at the fraction corresponding to the peak of galactosyl-transferase which is a marker enzyme of Golgi apparatus. This means both proteins are transported to Golgi apparatus. RER fraction of VμsM is larger than that of VμsE. This might reflect slower transport efficiency of VμsM protein. Compared with these two chimeric proteins, VμmM protein shows no peaks around Golgi and SER fractions.

There is another experimental method to show the transport of glycoprotein from RER to Golgi; an endoglycosidase H (endoH) method. An asparagine-linked glycoside attached to protein to be secreted or to be expressed on plasmamembrane was modified in Golgi apparatus from a high-mannose type to a complex type(Fig.5-11). EndoH can cleave high mannose type oligosaccharide but cannot cleave complex type. So we can determine the location of a glycoprotein by testing the resistance of the protein to endoH. Because in general, the rate limiting step of protein transport is the transport from RER to Golgi(Lodish, 1988), we can estimate the transport velocity by this method.

Cells were pulse-labeled with [^{35}S]-methionine for 20 minutes, chased for from 30 minutes to 4 hr's in a medium with cold methionine, solubilized, immunoprecipitated and digested with endoH. The fluorography of the SDS/PAGE is shown in Fig.5-12.

As a result, VμsE protein is transported very rapidly (less than 30 minutes) to Golgi. Compared with VμsE, VμsM is transported much more slowly (50% of the protein is transported after 4 hr's) and virtually no VμmM protein transport was detected. Therefore, a good correlation between membrane expression, subcellular localization, and transport velocity was demonstrated.

5.6. Reduction of negative charge in the acidic linker sequence of μm increases cell surface expression of the mutant μm chain

Because VμsM is found to be transported to the plasmamembrane, the importance of acidic linker has become obvious. To determine the crucial part of ER retention signal, site-directed mutagenesis of this region was done. As shown in Fig. 2-9, acidic charge in this region is relatively conserved. To reduce this charge, three glutamates were mutated to glutamines, which are also polar residues but the net charges are almost zero. Using a synthetic primer designed as in figure 5-13, an expression vector was made and designated pSV-VμmMQ3. This was transfected into J558L, selected, and tested for its surface expression of IgM. A slight increase in IgM-conjugated fluorescence was observed by flow cytometry (data not shown). So the cells were cloned and a representative clone with strong intracellular μ expression was selected. As shown in figure 5-14, the clone showed an obviously increased surface Ig expression compared with wild type mIgM transfectant with almost identical intracellular protein expression (not shown).

5.7. Transplantation of the acidic linker resulted in slower transport efficiency of other receptor

From the results presented until now, we can postulate that the acidic linker sequence has the ability to support retention of mIg molecule. To ascertain the ability of the sequence to retain other transmembrane molecule in ER, a transplantation experiment was done. The transmembrane protein we used was the interleukin-2 receptor α chain which is also called Tac antigen. Tac antigen was reported to be transported to membrane efficiently and used for similar transplantation

experiment (Bonifacino et al., 1990). The amino acid residues locating at similar location were replaced from Tac antigen by 11 aa acidic linker sequence (Fig. 5-15). After transfection of Cos-1 cell with each expression vector, transiently expressed protein on membrane was measured by flow cytometry. Cos-1 was used because it enables highly efficient transfection by calcium phosphate precipitation method and transient replication up to 1000 copies per cell of transfected plasmid which contains replication origin derived from SV40. The plasmids I used were pKCR-Tac2A (Fig. 5-16) and pKCR-Tac2AL-1, both of which encode SV40 replication origin together with SV40 promoter.

The result is shown in Fig. 5-17. From comparison of three histograms, it is clear that more than 10% of cells were transfected with each plasmid, i.e., the transfection efficiency is >10%. By comparing the mean fluorescence level of transfected populations of the latter two, it can be said that the average expression level of Tac2AL-1 protein on membrane is less than 1/2 of that of Tac2 protein because in this X axis in logarithmic scale 25.7 channels is equal to 2 fold.

5.7. Discussion

As shown above, sorting of chimera VμER overproducer resulted in acquisition of truncated receptor which lacked almost all the cytoplasmic region. We can give two explanations to this phenomenon. One explanation is that this is the result of proteolytic degradation. In fact, we could sometimes observe such degradation product after the immunoprecipitation procedure of VμMERΔCH2 protein (not shown). However, it can not explain an increased expression of the receptor of over 20 fold. The other explanation is that it is the result of spontaneous deletion of cytoplasmic gene segment of the receptor. The increased

expression is explained by stabilization of truncated receptor mRNA which is shorter than ever. In fact, artificial truncation of VμER cytoplasmic segment gave similar result.

Such lesion is observed naturally in the structure gene of insulin receptor of diabetes. In some insulin-independent diabetes, deletion of tyrosine kinase domain sequence from one allele was found (Taira et al.,1989, Odawara et al., 1989). This diabetes associated with high serum insulin inherits dominantly, which means truncation of one of two alleles shows dominant negative suppression of insulin receptor function. The possible reason for such dominant negative suppression is the increased expression of truncated receptor which prevents action of remaining normal receptor.

While the reason of the weak kinase activity detected from some clones that expressed truncated VμER receptor remains unclear, such truncation- increased expression mechanism may account for most of our present results.

Secreted proteins including membrane integral proteins are folded in lumen of RER before transported to Golgi apparatus. While folding, a RER resident protein Bip (GRP78) binds to hydrophobic part of proteins and is thought to help folding of proteins. One prominent characteristic of Bip is that it has a tag sequence in its C terminus to retain itself in ER called retention signal. It is KDEL (HDEL in yeast) in amino acid sequence which is common among lumenal ER resident proteins (Munro and Pelham, 1987).

For the transmembrane ER resident proteins with relatively longer cytoplasmic sequence, not strict ER retention signal is discovered but C-terminal consensus sequences are reported. They are -KXKXX or -XKKXX (Jackson et al., 1990). However, at least 10a.a. of cytoplasmic sequence is reported to be necessary for efficient retention.

In the case of membrane IgM, its cytoplasmic amino acids are only three (KVK) and it is not sufficient for retention by the mechanism described above. Moreover, retention of mIgM is tissue specific: in B (and preB) cell it is not retained in RER. Our aim was to discover the molecular apparatus for retention of this transmembrane molecule in tissue specific manner. The facts that the correctly folded secreted form IgM was efficiently secreted and VμsE protein which had EGF receptor transmembrane sequence was efficiently transported to membrane show that the sequence around mIgM transmembrane sequence was responsible for the retention. To dissect this region into two, a chimeric receptor was made and further mutation/transplantation analyses were done in regard to the acidic linker sequence which is at the N-terminus of transmembrane sequence.

According to our result described here, it is probable that glutamate-rich acidic linker sequence plays an important role in mIgM retention in myeloma. Hombach et al.(1990) concluded that lack of Ig-α expression was the primary reason of mIgM retention in myeloma by demonstrating that transfection of Ig-α gene restored mIgM surface expression in J558Lμm which expresses mIgM intracellularly. Ig-α has several positively charged residues at N terminal proximal to transmembrane region (Sakaguchi et al.,1988). So this region might interact with mIgM acidic region to make cell-surface complex. Also, tissue specific retention can be explained by the differential expression of Ig-α in different tissues. In fact Cos-1 fibroblast which does not express Ig-α transfected with both mIgM heavy and light chains was incapable of surface expression of mIgM (Williams et al.,1990).

While we focused our study on the acidic linker, mutation of charged residues in the transmembrane sequence was also reported to restore cell surface expression of mIgM (Williams et al.,1990). As a

conclusion, these two regions are thought to work synergetically to retain mIgM molecule. Though the precise mechanism of retention is as yet unknown, binding of Bip to transmembrane sequence would be a possible reason if the hydrophobic surface of transmembrane sequence is not buried in ER membrane due to the charged residues in or beside it.

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Figures

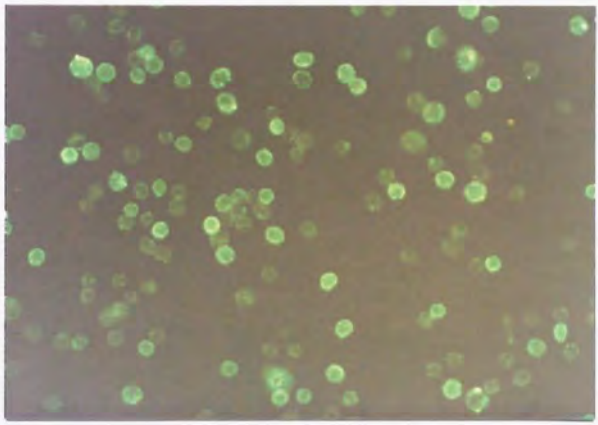


Fig. 5-1 Immunofluorescence microscopy of sorted J558L transfected with pSV-VμER. About 5% of positive transfectants were sorted using flow cytometric cell sorter EPICS753, and cultured for 2 weeks. Cells were surface-stained with biotinated anti-mouse Ig and FITC-avidin.

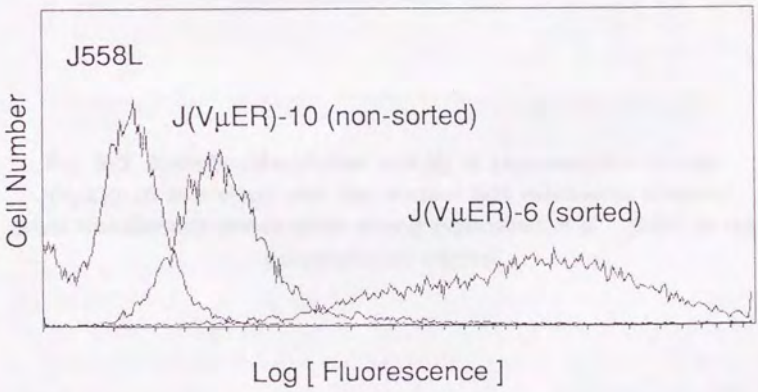


Fig. 5-2 Flow cytometric analysis of representative clones of sorted transfectants. 10^4 cells were analyzed for their surface IgM-conjugated fluorescence.

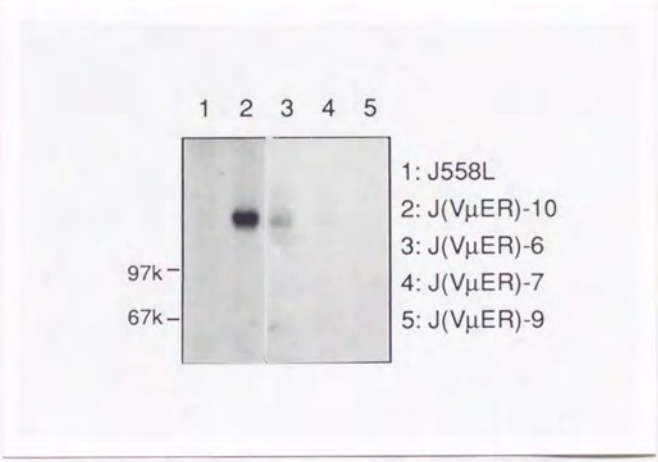


Fig. 5-3 Autophosphorylation activity of representative clones. J(VμER)-10 is a clone with low surface IgM expression whereas other transfectant clones show strong expression of it. J558L is the untransfected control.

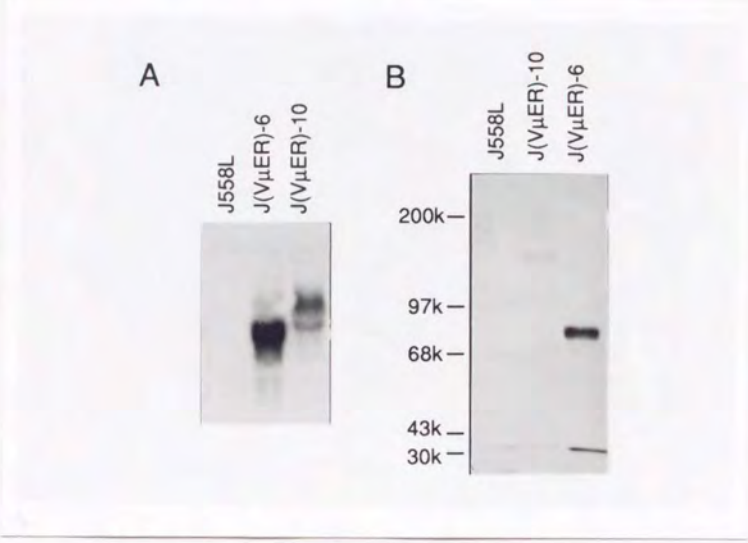


Fig. 5-4 A: Northern blot analysis of the clones. Total cellular RNA (10μg/lane) was blotted on nitrocellulose membrane and hybridized with [α^{32}P]-labeled IgM-specific probe. B: Immunoprecipitation of the metabolically labeled clones. Cells were labeled with [^{35}S]-methionine and the lysate was immunoprecipitated with anti-IgM together with protein A-Sepharose.

1 2 3 4 5 6 7 8 9 10

1 : J558L 4 -10: Serial dilution of J(VμER)-6
2 : J(VμER)-10
3 : J(VμErb) 10: 2^{-1}, 9: 2^{-2}, 8: 2^{-3}, 7: 2^{-4}, 6: 2^{-5}, 5: 2^{-6}, 4: 2^{-7}

Fig. 5-5 Comparison of intact and truncated receptor protein amounts. Immunoprecipitated protein was serially diluted and subjected to SDS/PAGE.

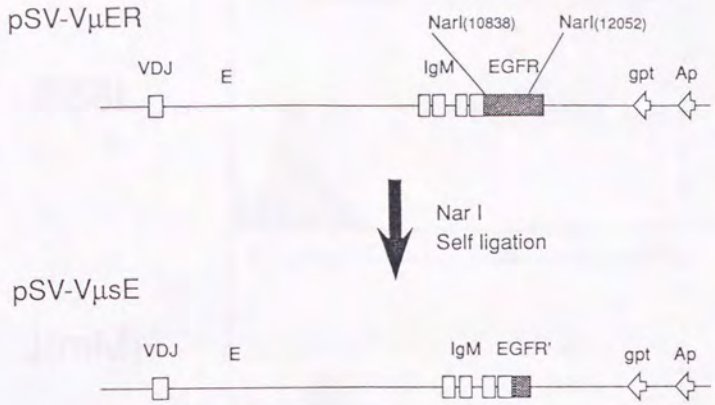


Fig. 5-6 Construction of pSV-VμsE

mM(wild type μm)

EGEVNAEEEGFENLWTTASTFIVLFLLSLFYSTTVTLFKVK

sM

KPTLYNVSLIMWTTASTFIVLFLLSLFYSTTVTLFKVK

sE

KPTLYNVSLISTGMVGALLLLLVVALGIGLFMRRRLD

: Transmembrane region

Fig. 5-7 Sequences of predicted carboxy-terminal amino acid residues of the receptor proteins.

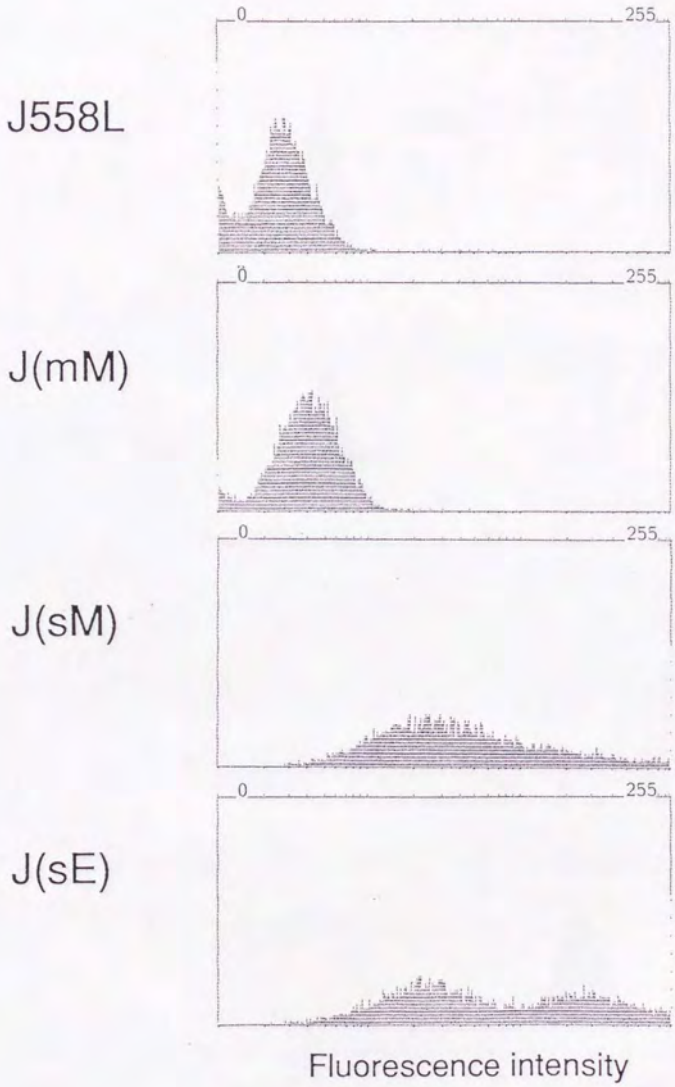


Fig. 5-8 Membrane expression of the receptors immediately after selection of transfectants.

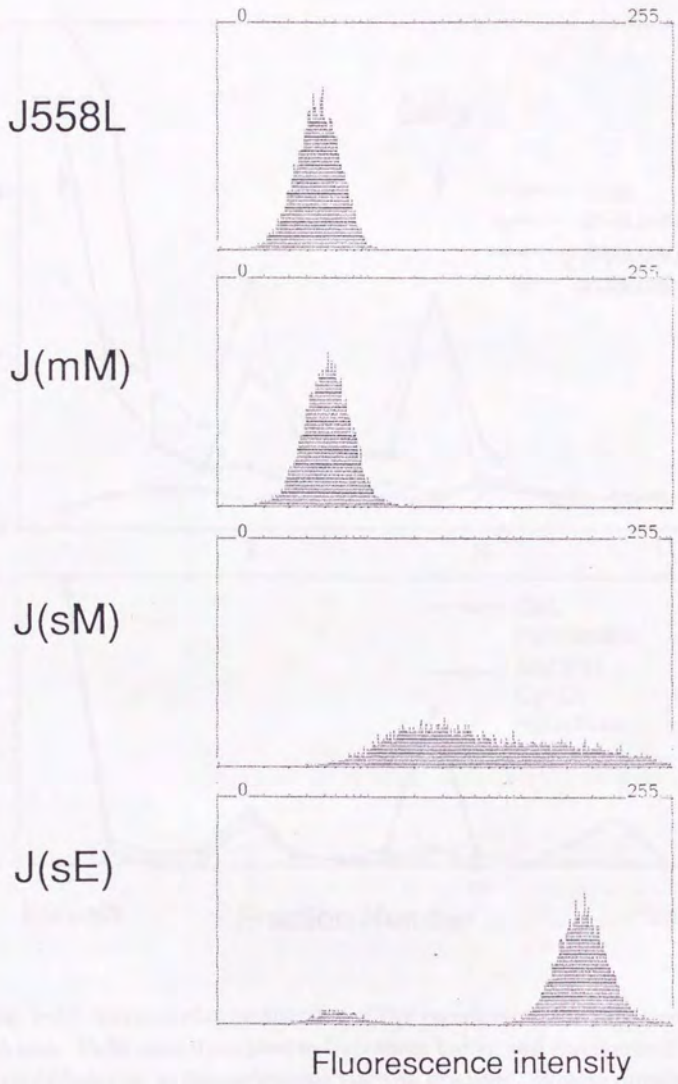


Fig. 5-9 Membrane expression of the receptors after cloning of representative clones.

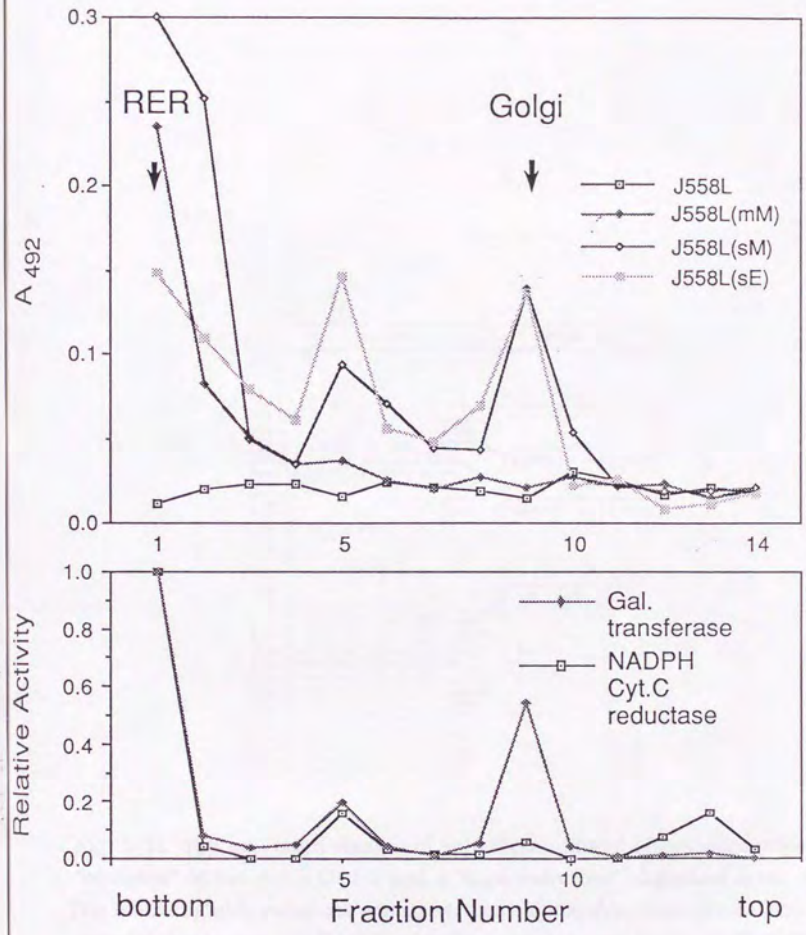


Fig. 5-10 Intracellular localization of the receptors in the representative clones. Cells were disrupted in hypotonic buffer and fractionated after centrifugation in discontinuous sucrose gradient. Protein amount in each fraction was measured by ELISA.

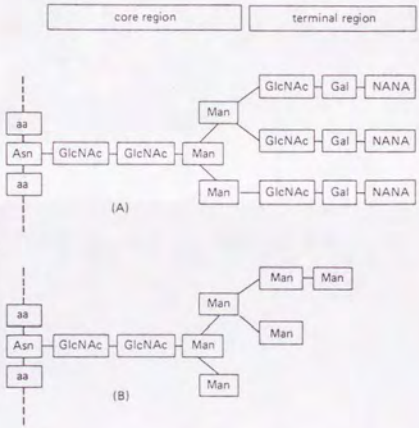


Fig. 5-11 The two main classes of asparagine-linked oligosaccharides: a "complex" oligosaccharide (A) and a "high-mannose" oligosaccharide (B). The following abbreviations are used: asparagine, Asn; mannose, Man; N-acetylglucosamine, GlcNAc; N-acetylneuraminic acid (sialic acid), NANA; galactose, Gal. (Alberts et al.,1983)

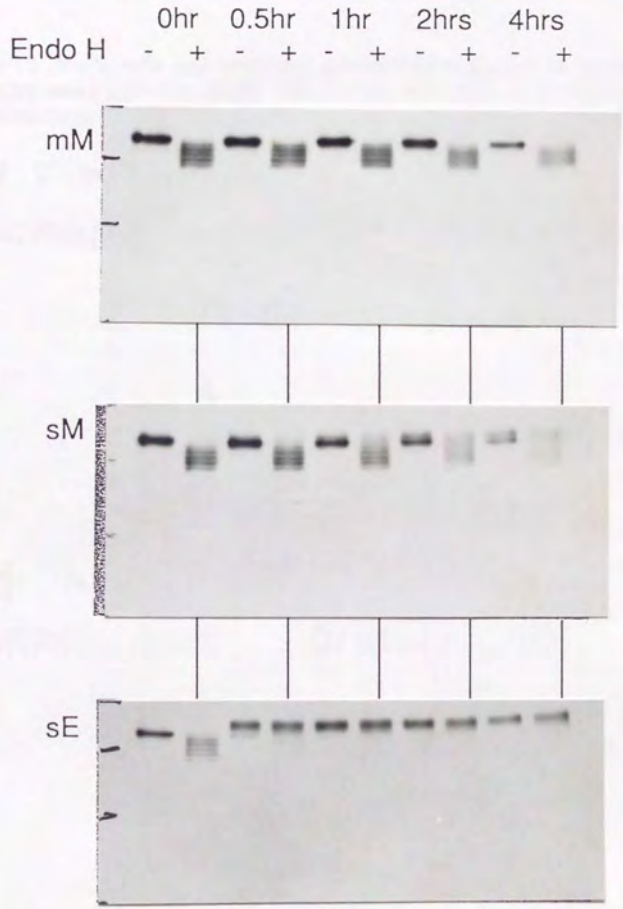


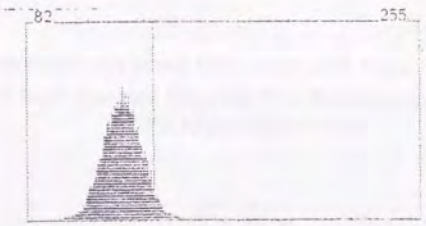
Fig. 5-12 Pulse-chase experiment to determine the rate of transport from RER to Golgi by monitoring acquisition of endoglycosidase H resistance. Cells were pulse labeled with [^{35}S]methionine for 20 minutes and chased for various periods shown above.

```
          V  N  A  E  E  E  G  F  E  N  L  W
W.T.  GGTGAATGCTGAGGAGGAAGGCTTTGAGAACCTGTGG
          V  N  A  Q  Q  Q  G  F  E  N  L  W
Q3    GGTGAATGCTCAGCAGCAAGGCTTCGAAAACCTGTGG
                                SfuI
```

Fig. 5-13 Nucleotide and predicted amino acid sequence of mutagenic primer to make pSV-VμmMQ3. The primer was used to introduce three point mutations to glutamates and a restriction endonuclease recognition site (SfuI).

mM(W.T.mIgM) :

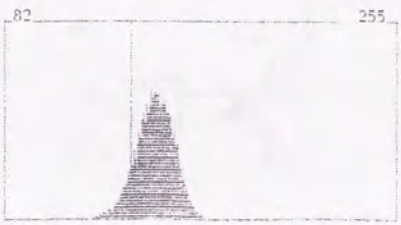
EGEVNAEEEGFE E:glutamic acid



5.71%

mMQ3: (to reduce charge)

EGEVNAQQQGFE Q:glutamine



89.51%

Fig. 5-14 Flow cytometric analysis of the wild type and mutant clones with almost identical receptor protein level. Amino acid sequences of acidic linker is shown above the figures. Percentage of brighter cell population above an arbitrary threshold is shown beside.

```
AatII                                          NaeI
------                                         ------
 ThrSerIlePheThrThrGluTyrGlnValAlaValAlaGlyCys
GACGTCCATATTTACAACAGAGTACCAGGTAGCAGTGGCCGGCTGT
       |                                       |
       GluGlyGluValAsnAlaGluGluGluGlyPhe
     CCGAGGGGGAGGTGAATGCTGAGGAGGAAGGCTTT
  TCGAGGCTCCCCCTCCACTTACGACTCCTCCTTCCGAAA
```

Fig. 5-15 Nucleotide and predicted amino acid sequence of synthesized linkers which were inserted into just 5' of transmembrane sequence of IL2Rα expression vector.

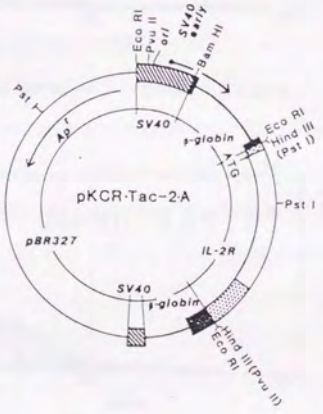
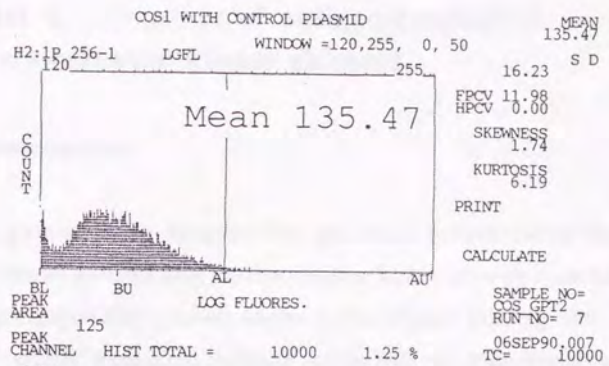
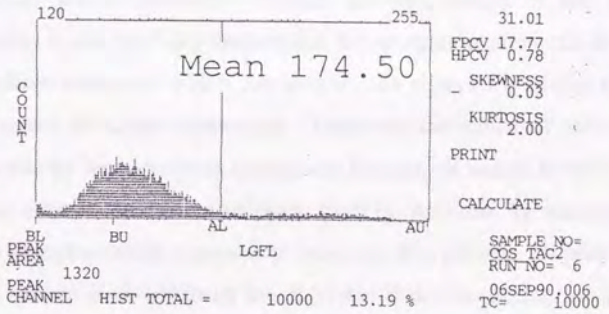


Fig. 5-16 pKCR-Tac2A

Mock:



pKCR-Tac2A:



pKCR-Tac2AL-1:

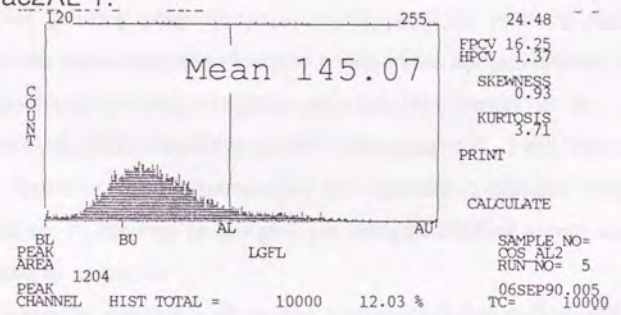


Fig. 5-17 Transient expression of wild type and mutant IL2Rα on Cos-1 plasmamembrane. Cells were transfected with each plasmid (Mock: pSV2gpt) ,incubated for 48hr's, stained with rat anti-IL2R (CD25) (Immunotech), biotinated donkey anti-ratIgG, and FITC-avidin. and analyzed by flow cytometry EPICS-C(CS) (Coulter).

Chapter 6. Creation of antigen-responsive antibody-receptor kinase chimera

6.1. Introduction

A growth factor receptor like epidermal growth factor receptor (EGFR) shows an inducible protein tyrosine kinase activity upon binding of the corresponding growth factor to its ligand binding site. The binding signal generated outside of a cell is transferred to the intracellular kinase domain. Though the mechanism of the signal transduction is not yet fully understood, the receptor system can be used as a sensitive biosensor which can amplify the signal originating from a small amount of target molecules. However the kinds of molecules recognizable by such receptor system are limited. It would be profitable if we can extend the system to every protein molecule by constructing chimeric receptor which responds to each specific protein. A possibility for such system is to construct the chimeric antibody joining the kinase portion of a receptor to an antibody. Until now, many chimeric antibodies joining other enzyme or polypeptides with its carboxy-terminus has been made and shown to possess both antigen binding ability and enzymatic or physiological activities(Neuberger et al., 1986; Williams et al.,1986; Schnee et al.,1987; Flanagan et al., 1988; Shin et al., 1990). However, these chimeras are not reported to regulate their own activities by its binding to antigen; i.e. antigen binding signal was not transduced to its partner.

To test the possibility of making such controllable molecule, I made chimeric receptors joining the extracellular and transmembrane portion of murine membrane type IgM (mIgM) with the cytoplasmic portion of human epidermal growth factor receptor (EGFR)(Ullrich et al.,1984;

Merlino et al.,1985). I made several chimeras and one of them showed antigen-dependent protein kinase activity.

6.2. Construction and expression of the chimeric receptor VμMER

Because previous version of chimera VμER showed relatively low kinase activity which seemed to be constitutive in its magnitude when antigen or anti-IgM was added (not shown), I decided to make another version of chimera. Since VμER had transmembrane sequence derived from EGFR, I made a chimera which had IgM transmembrane sequence. To make an expression vector, I combined IgM and EGFR cDNA using the splicing consensus sequence existing between M1 and M2 exons of IgM, because of the presence of a convenient restriction site *Pst*I. This was designated pSV-VμMER (Fig.6-1, 6-2).

This plasmid was introduced into murine myeloma J558L(Oi et al.,1983), and a highly expressive clone J(MER)-7 was selected as before. After metabolically labeling the clone, immunoprecipitations were done separately with anti-IgM and anti-EGFR antibodies. When the precipitates were analyzed, it confirmed the expression of polypeptides of Mr.~140kd and 30kd which coincide the expected M.W. of the chimeric receptor H and L chains (Fig.6-3).

6.3. Characterization of the chimeric receptor

Because the clones were selected by ELISA using NP-BSA as adsorbent, these molecules must have antigen binding site(s) composed of heavy and light chains. Indeed, polypeptides of similar molecular weight were able to be purified by NP-caproate-Sepharose column. After

purification, proteins were autophosphorylated *in vitro* and analyzed on SDS/5%PAGE at reduced or unreduced conditions (Fig.6-4). Whereas reduced sample showed phosphoprotein of similar molecular weight as immunopurified heavy chain, unreduced one showed significantly slower(~330kd) mobility which might be H_2L_2 form that was predicted from native mIgM structure.

To assess the possibility that this protein, we call VμMER, work as an immunosensor protein, lysate kinase assay was done. J(MER)-7 cells were harvested, lysed in lysis buffer, and mixed with antigen NP-BSA or rabbit anti-mouse IgM. The mixture was preincubated on ice, then added with [γ^{32}P]-ATP. When the gel was analyzed by autoradiography, it gave a novel band at around 140kd not found in J558L (Fig.6-5A). Because this band was resistant to alkaline treatment in 55°C 1M KOH for 2 hrs, it was considered to be tyrosine-phosphorylated (Fig.6-6). However, no significant alteration of the band density was observed for different preincubations. To test the validity of this assay, the same experiment using human epidermoid carcinoma A431 which overproduces EGFR was done (Fig.6-5B). Alteration of the density of a 170kd band which might be autophosphorylated EGFR was surely observed in my experimental condition.

6.4. Construction of chimeric receptors which cannot form heterotetramer

Because VμMER seemed to form H_2L_2 heterotetramer as mIgM and the oligomer formation of EGFR is considered to induce the kinase activity(Schlessinger,1988), I interpreted the result not in the way as the antigen binding did not induce the kinase activity but as the EGFR kinase activity was constantly activated. So I made a hypothesis; if the constant

heterotetramer formation of VμMER would be avoided, the kinase would be activated only when the chimeric antibody binds molecules which helps the oligomer formation of the chimera.

To forbid the constant heterotetramer formation of VμMER, the CH2 domain sequence was deleted which encoded a cysteine residue for inter-heavy chain disulfide bond from pSV-VμMER, and a new expression vector pSV-VμMERΔCH2 was made (Fig.6-1, 6-7). Transfection and the selection of a highly expressive clone J(MERΔCH2)-1 was done as before.

Immunoprecipitations with anti-IgM and anti-EGFR were done as before and the expression of polypeptides of Mr.~120kd and 30kd corresponding to the M.W. of the deleted heavy chain and λ1 light chain was confirmed (Fig.6-3). By estimation of anti-IgM purified band densities of the two clones, about 4 times larger amount of protein was expressed than VμMER protein in J(MER)-7. Affinity-purification and *in vitro* kinase assay was also done (Fig.6-3). Whereas reduced sample showed phosphoprotein of similar M.W. as VμMER, most of unreduced one showed a little larger M.W. than reduced one, which might represent HL heterodimer. It is noteworthy that the J(MERΔCH2)-1 lysate used for purification was four times of that of J(MER)-7, which means the specific autophosphorylation activity of VμMERΔCH2 measured so far is less than one tenth of that of VμMER.

To avoid heterotetramer formation of the chimera, two more versions of mutant were made (Fig.6-1). One was CH2-depleted chimera whose transmembrane segment was from EGFR (pSV-VμERΔCH2) and the other was a point mutant whose cysteine residue in CH2 was changed to alanine (pRSV-VμERCA) by using site directed mutagenesis. For this purpose, the expression vector based on RSV promoter was made to simplify the mutagenesis procedure (Fig. 6-7A, B). The expression

vectors were transfected to J558L and several clones were selected. According to autophosphorylation and PAGE analysis of the representative clone (Fig.6-4), the VμERΔCH2 protein was not stable and show very low kinase activity with unknown reason. VμERCA protein was rather stable, but in normal oxidative condition, considerable amount of protein was in higher molecular weight which might represent stable heterotetramer even in this denaturing condition.

6.5. Deletion of CH2 domain makes the receptor responsive to antigen

I measured the sensory function of the chimeric protein VμMERΔCH2 by lysate kinase assay. Simple preincubations without any addition or with BSA were taken as controls. When the reaction mixture was analyzed by SDS/7.5%PAGE, a novel band not found in J558L, with molecular weight corresponding to VμMERΔCH2 appeared (Fig.6-9A). Whereas the intensity of the control bands was reduced compared with J(MER)-7, their densities were significantly altered by anti-IgM or NP-BSA. To purify these bands, immunoprecipitation analysis of the half of reaction mixture by anti-IgM together with protein A-Sepharose was done. This gave similar change in band densities of up to 10 fold increase by anti-IgM (Fig.6-9B). In order to assess the dose dependency of NP-BSA, triplicate tests were made changing the concentration of NP-BSA during preincubation. The immunoprecipitated band density corresponding to autophosphorylation was found to increase linearly with concentration in lower concentration but attains the maximum at around 3μg/ml ($5x10^{-8}$M; $3x10^{13}$molecule/ml) (Fig.6-9C). This change in band densities by NP-BSA was also detected by immunoblot analysis using anti-phosphotyrosine antibody (Fig.6-10). Reaction mixture in which cold

ATP was used instead of [γ^{32}P]ATP was used for SDS-PAGE followed by Western blotting using either anti-IgM antibody or anti-phosphotyrosine monoclonal antibody PY20(ICN). While the 120kd band detected with anti-IgM was almost constant, the same band density probed with PY20 showed significant augmentation by NP-BSA preincubation.

6.6. Monovalent hapten inhibited activation by polyvalent antigen

These results lend support to the hypothesis that oligomer formation of the receptor by closslinking with antigen is essential for activation of the receptor kinase. In order to make a further check of the hypothesis, the effect of monovalent hapten was examined. When 50 fold molar excess monovalent hapten NP-caproate was added to the preincubation mixture with suboptimal concentration of NP-BSA, autophosphorylation was found to be severely inhibited (Fig.6-9C, 6-12). Also, about 5 times of optimal NP-BSA molar concentration of NP-caproate alone did not activate the kinase above basal level (Fig.6-12).

The effect of incubation time was also examined. Incubation time was taken as a parameter, and *in vitro* phosphorylation experiment was done(Fig.6-11,6-12). It was concluded that as short as 15 minutes was sufficient to activate the receptor kinase.

6.7. Substrate phosphorylation by purified receptor showed strange activation by certain antibody

To investigate the substrate phosphorylation activity of VμMERΔCH2 protein, effect of anti-IgM to the phosphorylation of

synthetic substrate poly Glu-Tyr by hapten-purified receptor was examined.

As hapten-purified receptors, VμMER, VμMERΔCH2, and VμER were used. VμERΔCH2 protein was not available because the expressed protein was very unstable and could not be obtained in enough amount. Purification was done batchwise with NP-Sepharose column and eluted with 1mM NP-caproate in HNTG buffer. Then the effect of addition of anti-IgM antibody was investigated with various concentration and with different origins. The level of peptide phosphorylation was quantitated by counting of TCA-insoluble radioactivity with liquid scintillation counter. As shown in Fig.6-13, preincubations of all the receptor proteins with rabbit polyclonal anti-IgM greatly enhanced peptide phosphorylation up to 20 fold. Preimmune rabbit Ig used as a control showed no such enhancement at all. The concentration dependence of activation differs a little at the middle concentration range between VμMERΔCH2 and other two.

To our surprise, such enhancement was not observed with goat anti-IgM antibody for either three receptors. So this enhancement is spieces specific and differ from the enhancement of autophosphorylation because CH2-containing receptors also can be activated.

Measured phosphorylation so far was thought to include both autophosphorylation and peptide phosphorylation. So the sample was applied onto electrophoresis instead of direct counting. As shown in Fig. 6-14, basal autophosphorylation of VμMERΔCH2 without anti-IgM seemed not apparently to be enhanced by the addition of anti-IgM. On the contrary, the extent of peptide phosphorylation was observed to be increased. So the different conditions such as the absence of other cellular components, for example protein phosphatase, or change in

detergent concentration (0.7->0.1% TritonX-100) might affect basal autophosphorylation of the receptor.

6.8. The efficient cell surface expression of VμMERΔCH2 protein

Membrane IgM is reported to be retained in RER and not transported to plasmamembrane in this cell line J558L as shown in chapter 5. In fact, VμMER producer J(VμMER)-7 expresses little cell surface IgM according to flow cytometric analysis (Fig.6-15). However, behind our expectations J(VμMERΔCH2)-1 showed far more surface expression than J(VμER)-10 which express a chimera with EGF receptor transmembrane sequence which allows efficient transport of the protein. This implies deletion of CH2 domain which is far from transmembrane sequence affects retention of protein in ER. To confirm this, a ΔCH2 version of membrane IgM which has only 3 cytoplasmic amino acids as wild type protein was made and expressed in J558L (Fig. 6-16). Flow cytometric analysis of the transfectant revealed a little rise of cell surface mIgM expression in transfectant (Fig.6-17). The fact that the level of expression was not so high implies deletion of CH2 domain together with mutation of cytoplasmic sequence allow release of firm blockage of the receptor protein in ER.

6.9. Discussion

I made several chimeric antibody-receptors and of one chimera succeeded in detection of *in vitro* antigen dependent kinase activity. Although several chimeric receptors of both prokaryotic and eukaryotic origins have been reported to transduce ligand binding signal to its signal

generation domain *in vitro* (Moe et al.,1989; Riedel et al.,1986) or *in vivo* (Riedel et al.,1987; Utsumi et al.,1989; Roussel et al.,1990; Lev et al.,1990; Yan et al., 1991), this may be the first report that the ligand binding domain is the antigen binding domain of antibody molecule. The construction of the gene of such antibody-receptor has to be made for each antigen. However, when it has done for an antigen, the works for other antigens are made easy because only the replacements of the variable domains are enough. The application of the polymerase chain reaction technology greatly facilitates the process (Orlandi et al.,1989; Sastry et al.,1989). The single-chain antibody will also be applicable (Davis et al.,1991).

For a rational design of our antibody-receptor, the knowledge on the signal transduction by antibody and the knowledge on the activation mechanism of the receptor are indispensable. We used membrane IgM because it is known to be expressed on B cell and deliver signals for its further differentiation. It is a constituent of a class of antigen receptor complex which is composed of two pairs of IgM H+L chains, pairs of accessory molecules (α and β)(Hombach et al.,1990; Campbell et al.,1991; Venkitaraman et al.,1991) and protein tyrosine kinase(s) *lyn* (Yamanashi et al.,1991). Number of tyrosine kinases including *lyn* and *blk*, G protein ($p21^{ras}$) and tyrosine phosphatase (CD45) are supposed to be involved in its signal transduction machinery (Dymecki et al.,1990; Campbell et al.,1990; Gold et al.,1990; Graziadei et al.,1991; Justement et al.,1991).

We used the epidermal growth factor receptor (EGFR) as our reporter domain because it is known to have ligand-inducible kinase activity as either in intact form or in chimeric form (Schlessinger, 1988; Ullrich & Schlessinger,1990; Riedel et al.,1986; Riedel et al.,1987). However, when we simply combined antibody with the intracellular

portion of EGFR, the kinase was found to be activated even in absense of the antigen. This result compelled us to have knowledge on the mechanism of activation of EGFR.

Oligomerization of cell surface receptor has been thought to be common activation mechanism because most of them can be activated by crosslinking with specific antibodies. In the case of EGF receptor, anti-EGFR induced aggregation of the receptor and DNA synthesis (Schreiber et al.,1983). Anti-EGFR Fab' fragment did not induced aggregation, but crosslinking of Fab' with anti-IgG antibody induced aggregation and DNA synthesis. Similar observation was reported in the case of insulin receptor (Kahn et al., 1978). Based on these abservations we took up our work.

However, precise molecular mechanism of receptor activation has not been clear until recently. Dimerization as activation mechanism of receptor tyrosine kinases was proposed based on *in vitro* experiment (Yarden & Schlessinger, 1987) and *in vivo* experiments (Honegger et al., 1989; Kelly et al.,1991; Kashles et al., 1991; Blume-Jansen et al., 1991; Li & Stanley; 1991). In principle, receptor dimerization leads to cross-phosphorylation of each tyrosine residue in its carboxy-tail and the phosphorylated tyrosines serve as binding sites for the signal transfer particle containing proteins having homologous region with oncogene *src* (SH2 and SH3)(Wahl et al.,1988; Margolis et al.,1990; Ellis et al.,1990; Bjorge et al.,1990; Pignataro et al.,1990; Koch et al.,1991 and references therein).

Based on this dimerization hypothesis, I made the VμMERΔCH2 by deleting CH2 region from the simple antibody-receptor VμMER. It showed the mode of activation somewhat similar to that of EGFR. If we rely on the dimerization strategy, we may use other enzymes which function in dimer. For example, restriction endonuclease which works in

dimer may be utilized for the purpose, although more careful design would be necessitated than the present case. Use of fluorescence as reporter signal may be attractive in practical application. The recent work by Adams *et al.* (1991) who detected active cAMP-dependent protein kinase *in vivo* using two fluorescent dyes is very suggestive in this sense.

The reason of bimodal phosphorylation of our chimera which has optimal concentration for maximum activation is not clear. However, recent reports about platelet-derived growth factor receptor activation *in vitro* (Heldin et al.,1989) and chimeric human growth hormone - granulocyte colony-stimulating factor receptor activation *in vivo* (Fuh et al.,1992) show such bimodal activation pattern of receptors those are activated by ligand-induced dimerization.

It is possible that excess ligands may prevent the receptor from dimerization and make them monomeric inactive form. According to the estimation by ELISA, the concentration of VμMERΔCH2 protein in the lysate was in the range of several μg/ml (not shown). It is possible when equimolar amounts of a antigen and two receptors form complex, the maximum activity is attained. In that case, while the Kd of the original antibody for NP-cap is 1.2μM (Jones et al.,1986), when we assume the same Kd for the chimera, only a few percent of antigen is complexed with the chimera[1]. It means that using antibody gene with higher affinity, we may be able to get more complex and higher sensitivity.

The antigen dependent activation of tyrosine kinase was thus confirmed *in vitro*. Although the *in vitro* phosphorylation *per se* can be utilized for my purpose, the *in vivo* activity is desirable to attain higher

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[1]When the bound antigen concentration is x and we assume antigen-antibody complex formation of 1:2,

$$\frac{(5\times10^{-8}-x)(5\times10^{-8}-2x)}{x}=1.2\times10^{-6}$$

$x=1.86\times10^{-9}$[M]. The percentage of complexed antigen is 3.7%.

sensitivity. The *in vivo* test is also necessitated to confirm the validity of the dimerization hypothesis in the cell.

I made the *in vivo* test. The myeloma J558L transfected with pSV-VμMERΔCH2 and the fibroblast NIH3T3 transfected with VμERΔCH2 expression vector based on RSV promoter (Gorman et al.,1982) were analyzed for the antigen-dependent tyrosine phosphorylation *in vivo* by immunoblot analysis with anti-phosphotyrosine antibody. We have not yet succeeded in the detection of the antigen-dependent activation of the kinases. Even in the absence of antigen, a sufficient phosphorylation was observed and the level was not significantly affected by the addition of the antigen (data not shown).

A similar experiment was performed by Flanagan *et al.* (1988). They transfected NIH3T3 with IgM/*neu*, a fusion gene between IgM and the oncogene *neu* which was a highly homologue of EGFR. When the cell was cotransfected with the gene for immunoglobulin light chain, the heavy chain-neu peptide was expressed at the cell surface. And in the absence of the antigen, the cell was found to be transformed. This corresponds to our experiment without the antigen. The only difference is that their chimera retains CH2 domain and is supposed to form a heterotetramer. Therefore it is acceptable that their chimeric protein, when expressed at the cell surface, had active kinase and leads to transformation even in the absence of antigen.

The constitutive phosphorylation of the chimeric antibody in the absence of antigen is beyond the explanation of my model. A possible reason is the increase of efficiency of dimerization when the chimeric receptors are aligned on a cell membrane. As Schlessinger has suggested (1988), when a finite number of receptors are aligned on a membrane, the local concentration will be higher than they are dispersed in a volume (Schlessinger et al.,1978; Zidovetzki et al.,1986). The increased

concentration together with reduced mobility will enhance the chance of dimer formation of the chimeric antibody even in the absence of antigen.

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Figures

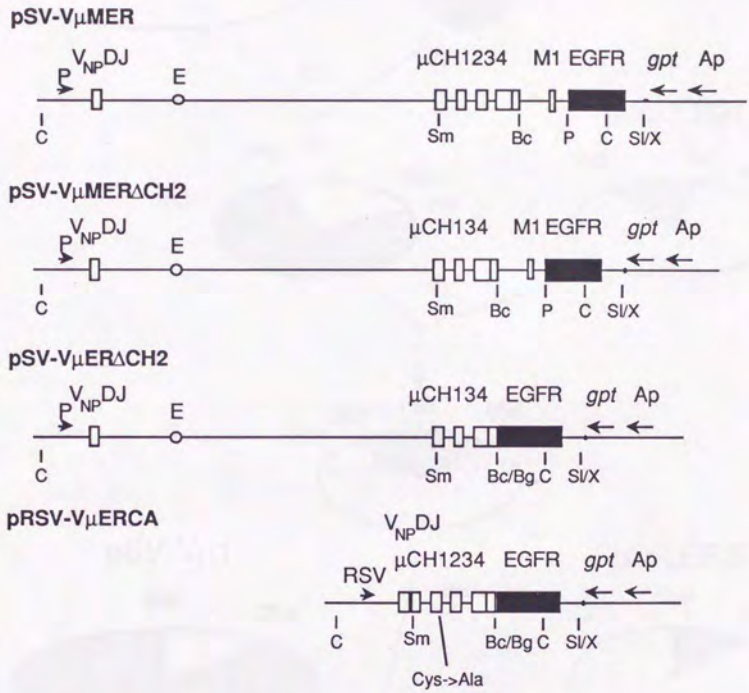

Fig. 6-1 Expression vectors made and used in this chapter. P: Immunoglobulin(Ig) promoter; E: Ig enhancer; gpt: mycophenolic acid resistance; Ap: Ampicillin resistance; Small circle: polyadenylation site; RSV: RSV promoter and enhancer; Restriction sites shown are C: ClaI; Sm: SmaI; Bc: BclI; Bc/Bg: BclI/BglII; P: PstI; Sl/X: SalI/XhoI. EGFR sequence for a.a. 661-1184 is included in pSV-VμMER and VμMERΔCH2, and a.a. 624-1184 is included in latter two vectors.

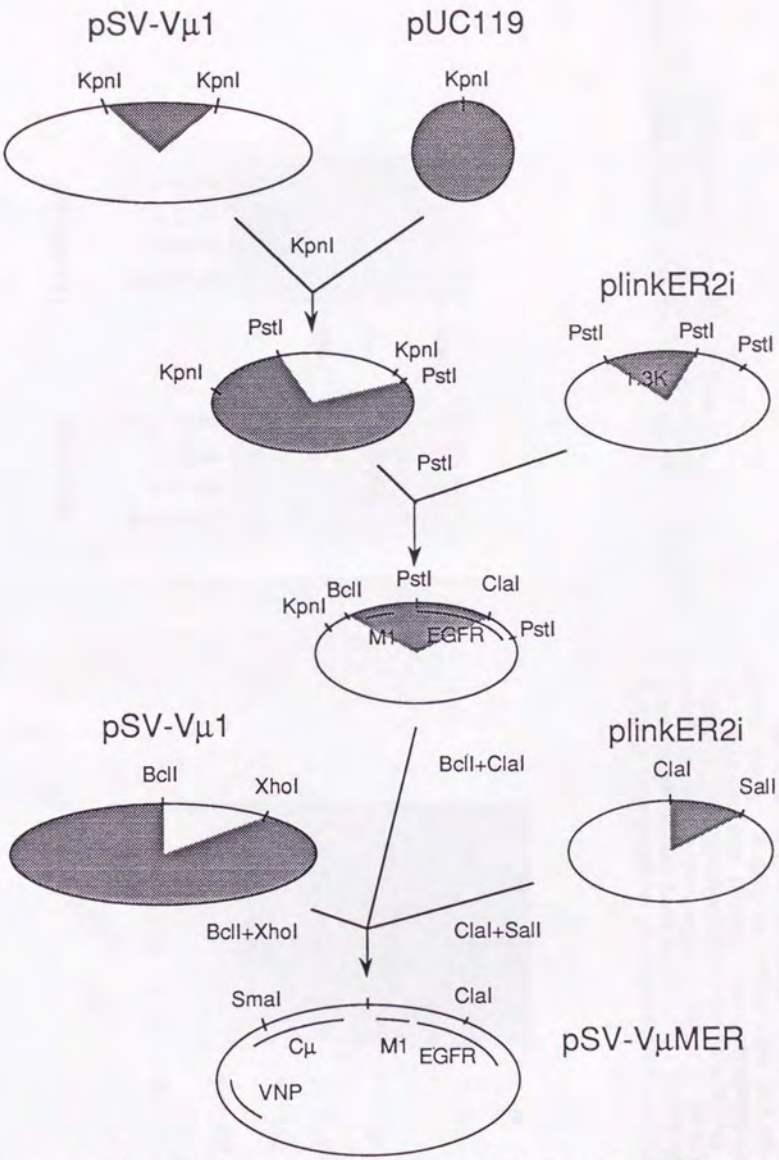

Fig. 6-2 Construction of pSV-VμMER. Hatched region is used for the next construct.

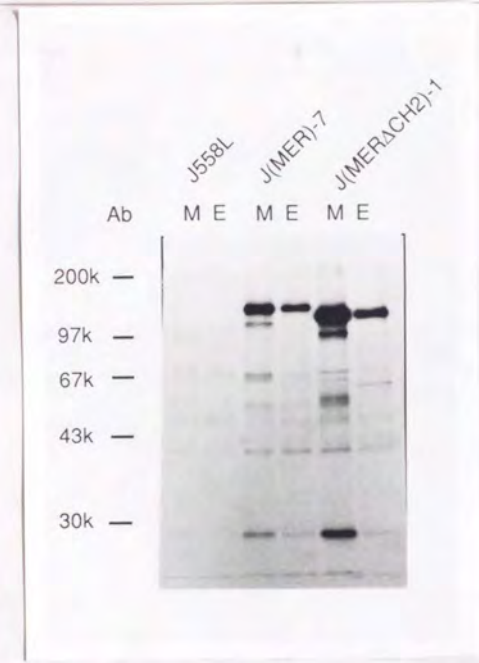

Fig. 6-3 Immunoprecipitation of metabolically labeled transfectant clones. Antibodies used are M: rabbit anti-mouse IgM(Zymed); E: rabbit anti-human EGFR cytoplasmic domain. The clones were metabolically labeled with [^{35}S]methionine, lysed, divided into two and immunoprecipitated with either antibodies. Equal counts of TCA insoluble lysates were used per sample.

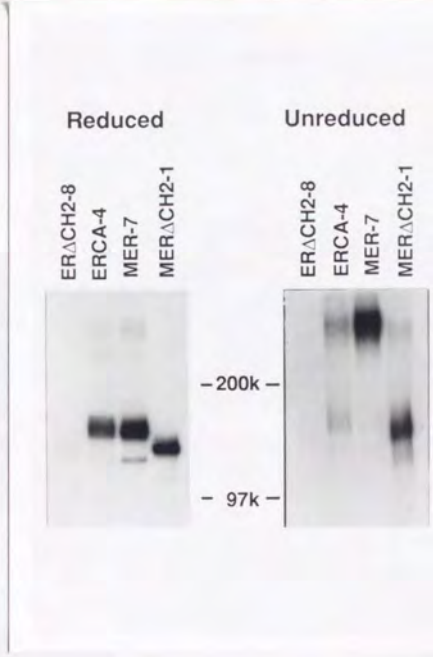

Fig. 6-4 Autophosphorylation of affinity purified hybrid receptors. Samples are electrophoresed on SDS/5% PAGE at reduced or unreduced conditions.

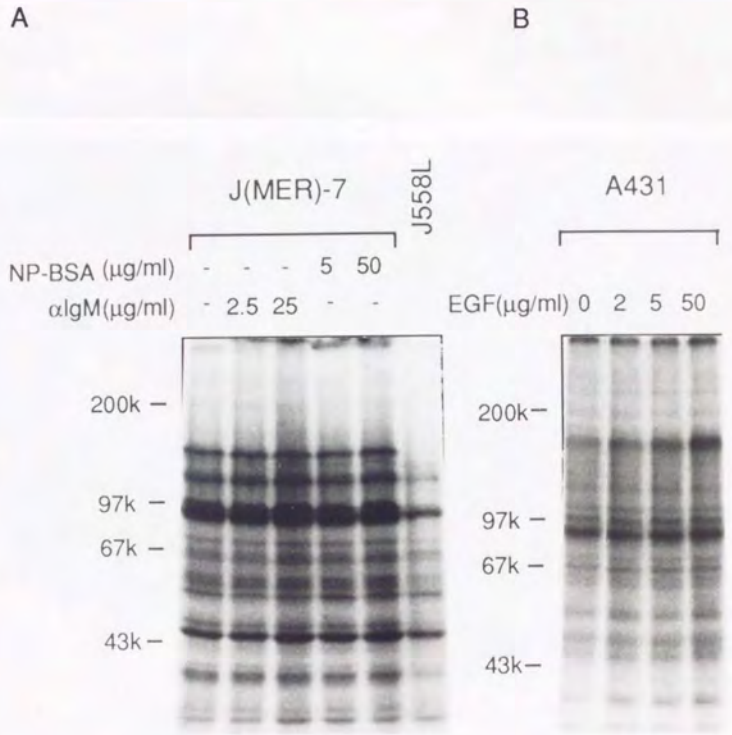

Fig. 6-5 A: Lysate kinase assay of J(MER)-7. ~2x10^6 cells per lane were lysed and mixed with indicated reagents with final concentration on ice for 120 min. J558L cell lysate without any addition was taken as a control. B: Lysate kinase assay of A431. Mouse EGF (Takara shuzo) at final concentration as indicated was added to A431 lysate 30 min prior to kinase reaction .

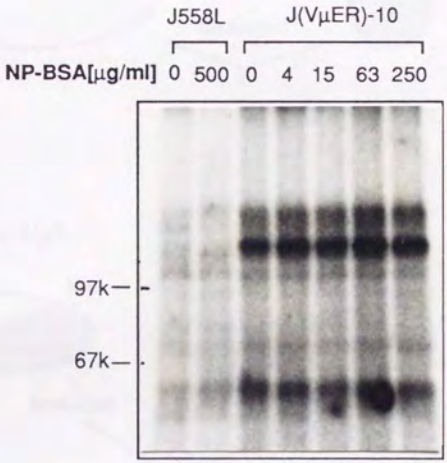

Fig. 6-6 Autoradiography of alkaline treated gel of J(VμER)-10 lysate kinase assay. The gel was treated with 1M KOH at 55°C for 2 hrs prior to acid washing and drying. Phosphoserine and phosphothreonine is known to be very labile in this condition. An intensely phosphorylated band seen above 97kd in the lanes of J(VμER)-10 seems to be tyrosine-phosphorylated VμER protein.

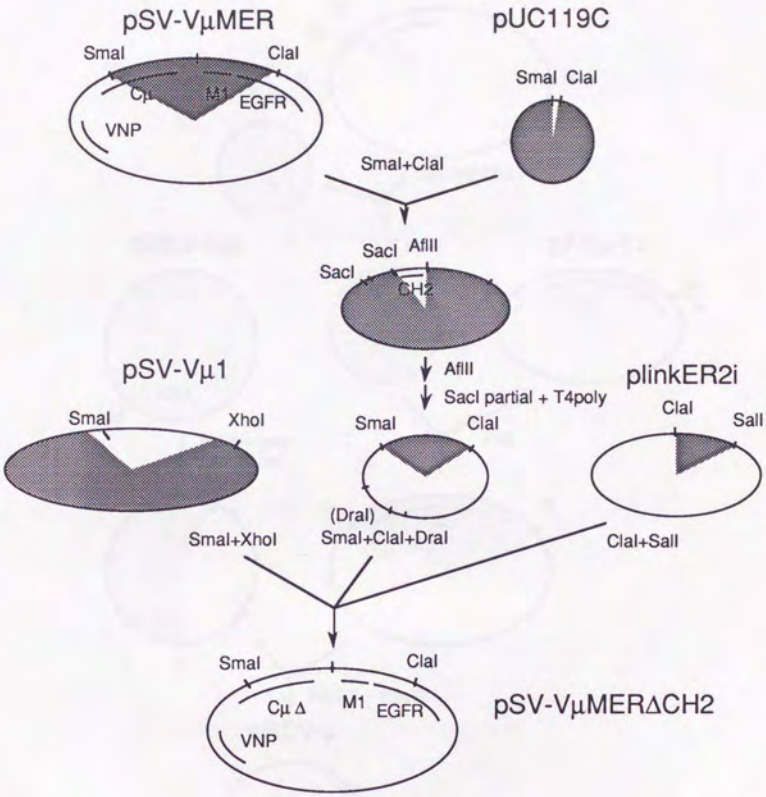

Fig. 6-7 Construction of pSV-VμMERΔCH2

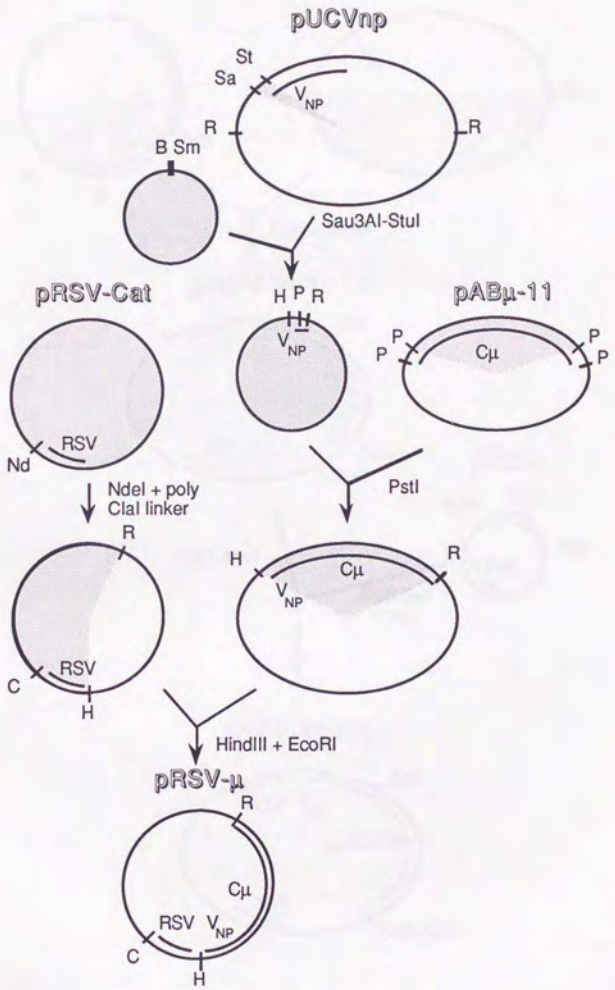

Fig. 6-8A Construction of pRSV-VμER {1} Construction of pRSV-μ.

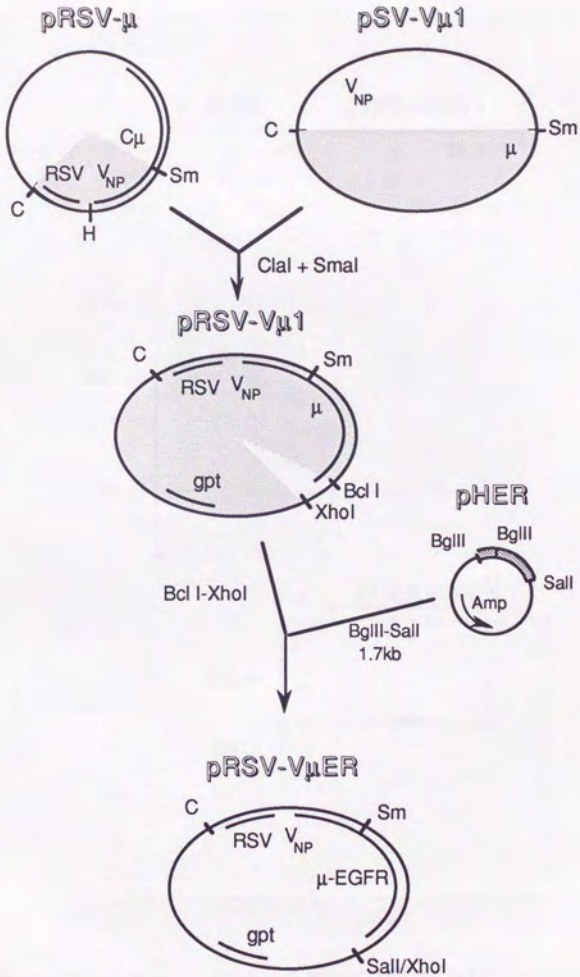

Fig. 6-8B Construction of pRSV-VμER {2}

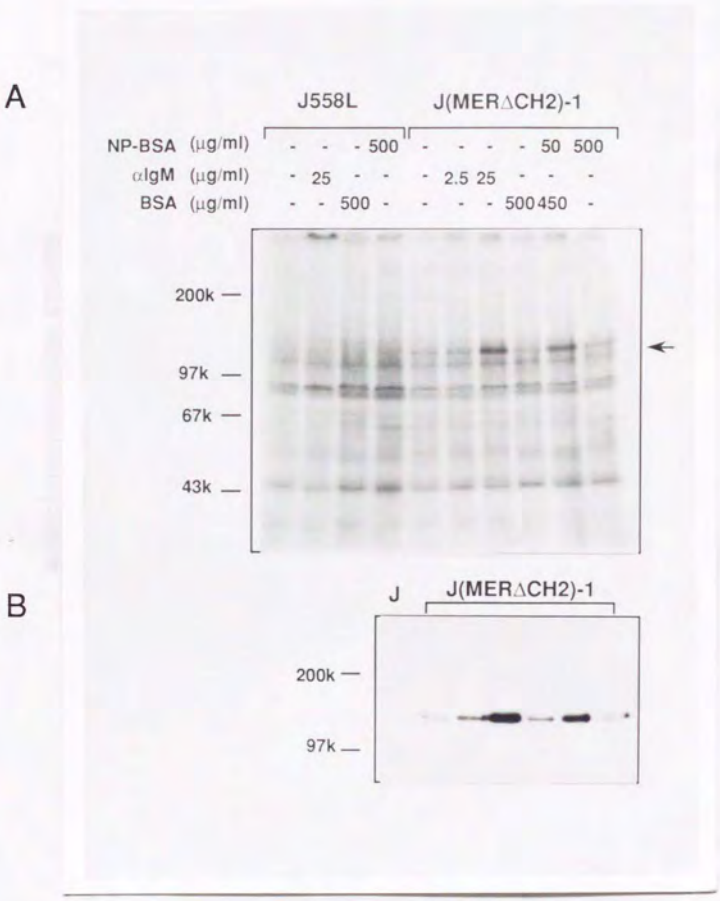

Fig. 6-9 A: Lysate kinase assay of J(MERΔCH2)-1 {1} Conditions are the same as in figure 6-5A.; B: Immunoprecipitation of *in vitro* phosphorylated lysate. Immunoprecipitation of auto-phosphorylated receptor was done with 5μg rabbit anti-IgM and 3mg protein A-Sepharose per sample. J558L lysate without any addition was taken in lane J.

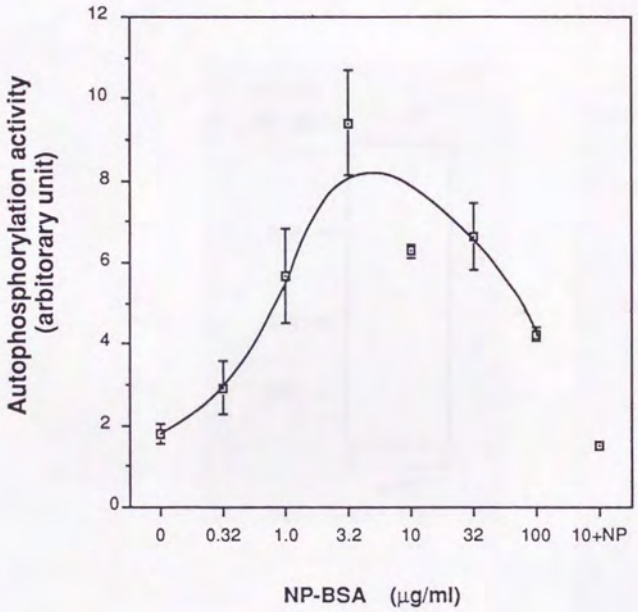

Fig. 6-9C NP-BSA dose dependency of autophosphorylation of MERΔCH2 protein. In lane 10+NP, final 50μM NP-caproate together with final 10μg/ml NP-BSA was added. Several independent experiments were done and a representative result is shown.

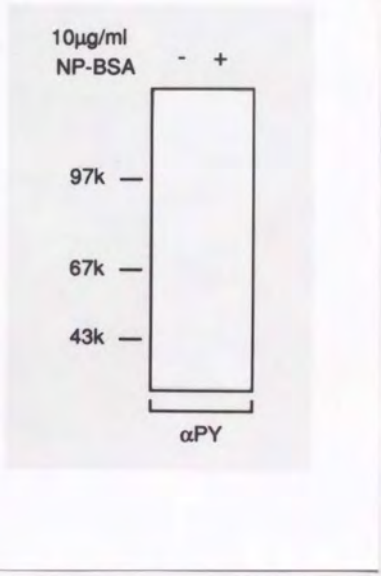

Fig. 6-10 Lysate kinase assay of J(MERΔCH2)-1 {2} Detection by Western blotting. J(MERΔCH2) lysate was incubated with/without 10μg/ml NP-BSA on ice for 20 min., kinased with cold ATP for 10 min at 37°C, and analyzed with Western blot using anti-phosphotyrosine Ab PY20 (ICN) (mIgG2b) + peroxidase conjugated anti-mouse IgG2b (Zymed). αPY means anti-phosphotyrosine antibody.

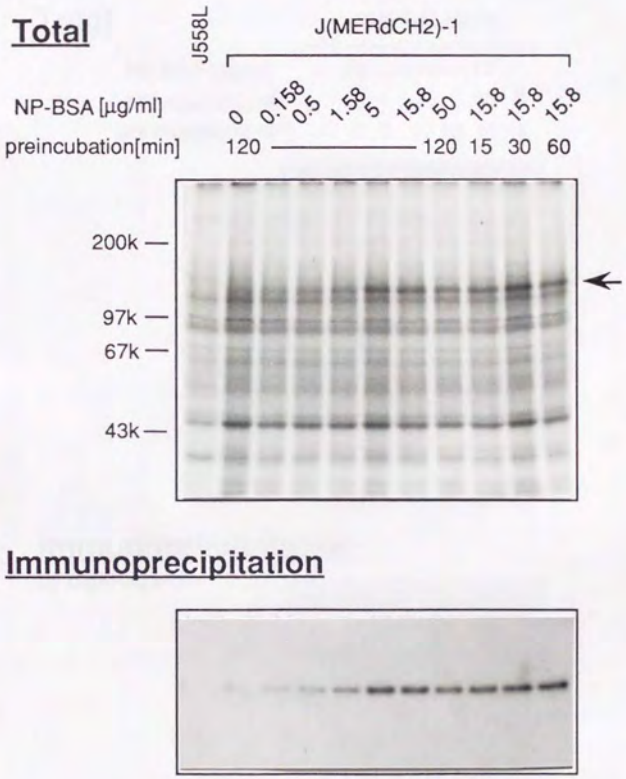

Fig. 6-11 Lysate kinase assay of J(MERΔCH2)-1 {3} Effect of antigen-concentration and preincubation time.

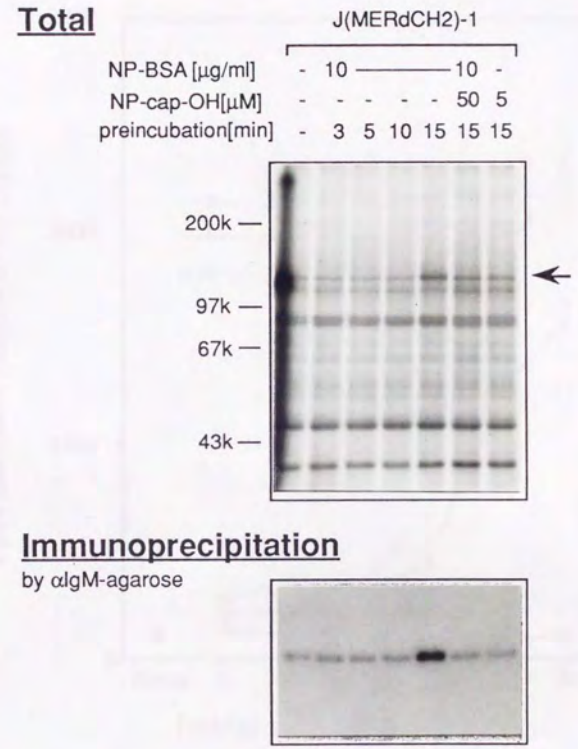

Fig. 6-12 Lysate kinase assay of J(MERΔCH2)-1 {4} Effect of preincubation time and free hapten NP-caproate on activation.

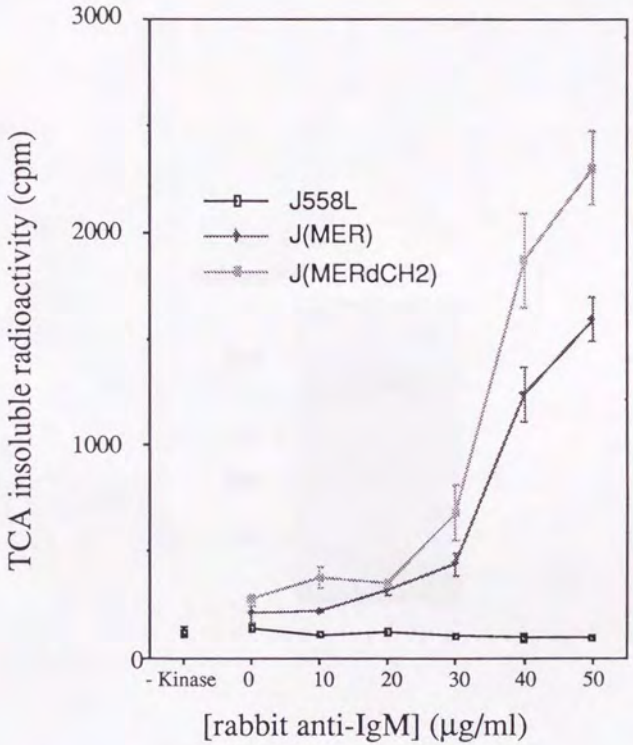

Fig. 6-13 Stimulation of exogenous substrate phosphorylation activity of purified protein by rabbit anti-IgM. Affinity-purified receptor was mixed with rabbit anti-IgM on ice for 15 min., then 1 vol. of reaction mixture containing 0.1mg/ml poly GluTyr and 50μM [γ^{32}P]ATP was added and incubated for 10min. 37°C. Peptide-incorporated radioactivity was recovered by TCA.

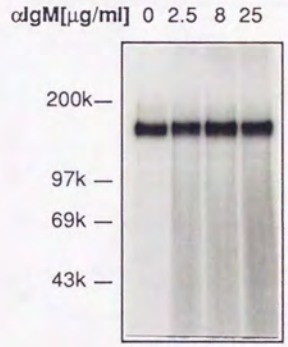

Fig. 6-14 Autoradiography of PAGE of peptide phosphorylation mixture. Experimental protocol was almost the same as in Fig. 6-15 except that reaction mixture was applied to SDS/PAGE instead of Whatman 3MM paper.

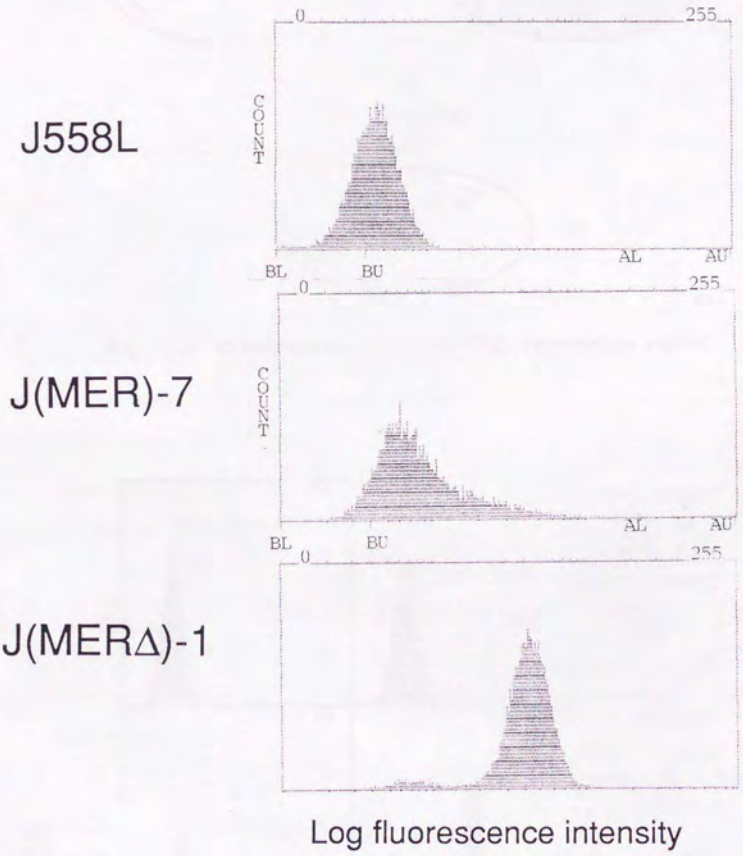

Fig. 6-15 Flow cytometric analysis of transfectant clones which express chimeric receptor with mIgM TM.

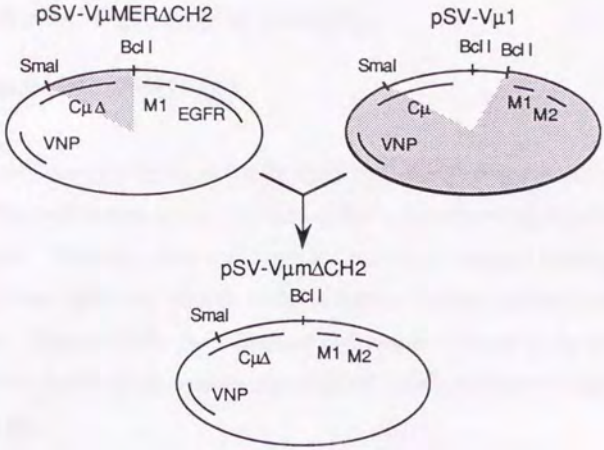

Fig. 6-16 Construction of mIgMΔCH2 expression vector

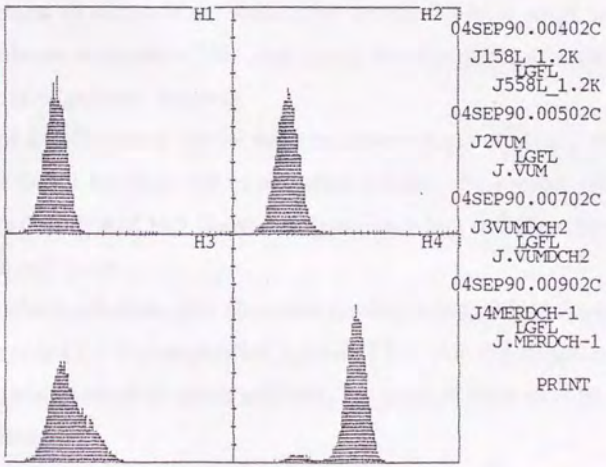

Fig. 6-17 Flow cytometric analysis of H1: J558L H2: J558L(Vμm)
H3: J558L(VμmΔCH2) H4: J558L(VμMERΔCH2)-1

Chapter 7. Concluding remarks

7.1. Conclusions of this study

I will summerize my research about IgM-EGFR chimeric receptor.

1. The expression system of IgM-EGFR chimera using myeloma was elucidated. The expressed molecule retained both antigen binding ability derived from IgM and protein tyrosine kinase activity derived from EGF receptor. Unexpectedly the expressed chimera was found to be efficiently transported to the plasmamembrane of J558L myeloma where mIgM cannot reach at all.
2. Sorting of cell surface IgM-EGFR-rich cell by flow cytometry yielded cells with truncated receptor which lacked cytoplasmic region.
3. Artificially truncated IgM-EGFR (sE) was efficiently expressed and transported to J558L plasmamembrane. A comparison between mIgM and sE was done to discover the molecular structure which helps to retain transmembrane molecule in ER. As a result, the importance of acidic linker sequence in mIgM was observed.
4. IgM-EGFRs which cannot form heterotetramer were found to mimic the native EGFR structure and its activation scheme. As a result, one of the constructs (VμMERΔCH2) showed antigen-dependent autophosphorylation activity in cell lysate.
5. Synthetic substrate poly glutamate-tyrosine was anti-IgM dependently phosphorylated by hapten-purified IgM-EGFRs. All the receptors tested showed the activation by rabbit antibody, but none of them showed that by goat antibody.

7.2 Prospects to future

IgM-EGFR as a model of chimeric antibody with antigen-responsive enzymatic activity is the first step to elucidate the protein engineering of the chimeric sensory macromolecule. We can imagine various type of such molecules which can be activated by dimer formation as described in the discussion of chapter 6. As discussed, all the allosteric enzymes which is active in dimer are potential targets of fusion partner. More stable and easy to measure chimeric Ig-enzyme will surely be obtained in the future when we can use more detailed structural information about our target enzymes.

Although the effect of antigen to IgM-EGFR *in vivo* remains unclear, the tyrosine kinase apparently plays the crucial role in receptor-mediated signal transduction and transformation of the cells. The observation obtained so far strengthen the hypothesis of receptor dimerization as the activation mechanism of receptor-type tyrosine kinase(RTK). Further experiments using various chimeric receptors or receptor mutants will unvail complicated signal transduction pathways originated from RTKs. For example, fibroblast growth factor (FGF) receptor whose auto-phosphorylation site Y766 was mutated to phenylalanine was reported to fail to associate with PLCγ in response to FGF, lack PtdIns hydrosis and Ca^{2+} mobilization after FGF stimulation, but still mediate mitogenesis in response to FGF (Peters et al., 1992; Mohammadi et al., 1992). Similar observations were made for native CSF-1, IGF-1, and insulin receptors (Ullrich and Schlessinger, 1990). As authors stated, this mutant might be an useful tool for dissecting the role of PtdIns hydrosis in cellular responses mediated by RTKs.

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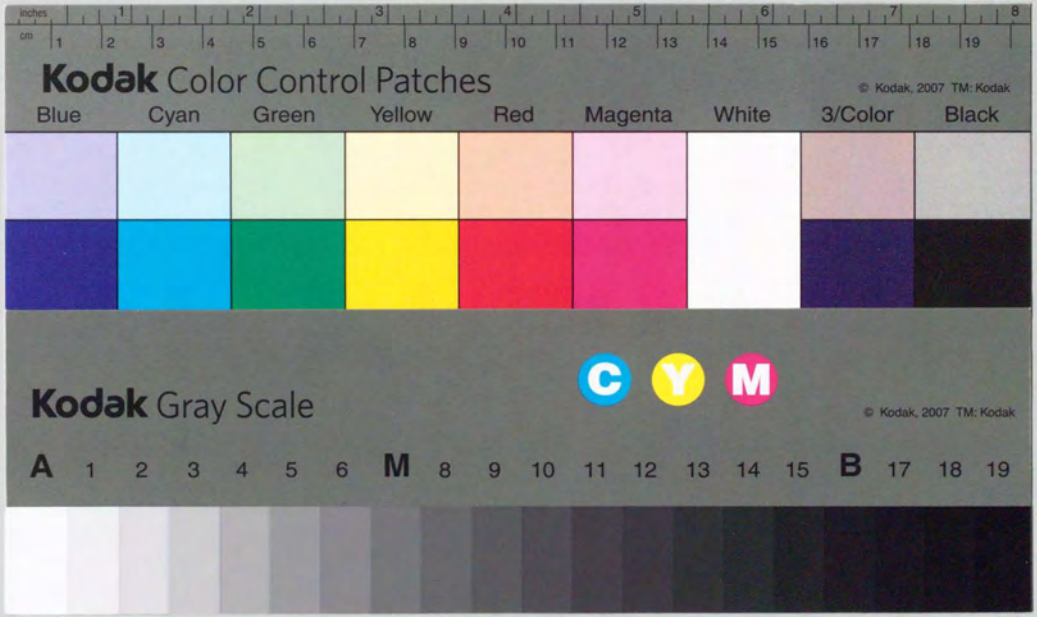
Whose Protein Kinase Activity Can Respond To Antigen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