Reconstitution of the epidermal growth factor receptor in artificial lipid bilayers

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Epidermal growth factor receptor, isolated in the presence of the non-ionic detergent octylglucoside, was reconstituted into liposomes by detergent dialysis. The receptor was shown to be incorporated in the lipid bilayer with the EGF binding domain oriented to the outside of the liposomes and to retain its ability to bind EGF and an anti-receptor antibody in a specific manner.

Epidermal growth factor receptor *Reconstitution* *Liposome* *Octylglucoside*

1. INTRODUCTION

The EGF receptor is a 170-kDa membrane-spanning glycoprotein which is able to bind EGF with high affinity and specificity. Upon binding, a variety of responses occur including clustering and internalization of EGF-receptor complexes, changes in ion fluxes, phosphorylation of cellular proteins and of the receptor itself at tyrosine residues and finally, upon prolonged stimulation (6–8 h), DNA synthesis occurs and the cells divide [1–3]. The amino acid sequence of the receptor has been deduced and the transmembrane and cytoplasmic domains found to be closely related to the v-*erb-B* oncogene product of avian erythroblastosis virus [4]. The EGF binding activity is located in the extracellular portion of the molecule whereas the protein kinase activity is in the intracellular domain.

Purification of the EGF receptor demands extraction of the molecule from the membrane bilayer with detergents and so far studies on the isolated receptor have been conducted under these non-physiological conditions. We report here the reconstitution of purified receptor into artificial phospholipid bilayers using a detergent-dialysis method. We provide evidence for a uniform orientation of the receptor within the bilayer with the EGF binding domain facing outside the liposomes and show that the incorporated receptor is functional in binding EGF and a monoclonal anti-receptor antibody. This reconstitution system should prove useful for the study of the structure and function of the EGF receptor in a more physiological environment.

2. MATERIALS AND METHODS

Octylglucoside, phosphatidylcholine (PC), phosphatidylethanolamine (PE), cholesterol, protease (from *S. griseus*), bovine serum albumin, phenylmethylsulfonyl fluoride (PMSF), rhodamine isothiocyanate (RITC) and protein A-Sepharose were from Sigma. [^{35}S]Methionine (1400 Ci/mmol) and Na^{125}I (15.1 mCi/mg) were from Amersham. All other chemicals were from BDH.

Epidermal growth factor (EGF) was prepared from mouse submaxillary glands as described [5]. EGF receptor was purified from A431 cells (grown in DMEM/10% foetal calf serum) as described [6], with the exception that octylglucoside (50 mM) was used instead of Triton X-100 or deoxycholate during elution of the protein from the EGF-affinity column. Radiolabelled receptor was

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prepared in the same way from A431 cells incubated for 18 h with 30 μCi/ml [^{35}S]methionine. The mouse monoclonal anti-EGF receptor antibody (EGFR1) was a kind gift of Dr M.D. Waterfield (ICRF, London). Both EGF and EGFR1 were radiolabelled with ^{125}I as described in [7].

SDS-polyacrylamide gel electrophoresis was performed as described [8]. Visualization of ^{35}S-labelled proteins after impregnation of gels in 1 M sodium salicylate was by fluorography on preflashed Kodak XAR 5 film at -70°C [9].

Reconstitution: A mixture of PC, PE and cholesterol (2:1:1, w/w) in 95% chloroform-5% methanol was dried on the walls of a polypropylene tube, redissolved in ether and redried twice under nitrogen with vortex-mixing to produce a thin film. The lipid film was then dried under vacuum in a Speed-Vac concentrator for 2 h to remove traces of solvents. The mixture was dissolved in Tris-buffered saline (TBS: 20 mM Tris, 150 mM NaCl, pH 7.4) containing 50 mM octylglucoside. EGF receptor in 20 mM Tris, 50 mM octylglucoside, 5 mM ethanolamine, 10% glycerol, 50 mM benzamidine, pH 8.0, was added and mixed by vigorous shaking or sonication for 10 min. The final detergent-to-lipid ratio was 40:1 (w/w) and the final protein-to-lipid ratio 1:100 (w/w). The solution was dialysed for 36 h against 3 changes of TBS containing 30 mM benzamidine and 0.1 mM PMSF. The resulting turbid suspension was either centrifuged at 100 000 × g for 1 h to collect the liposomes or mixed with sucrose to 40% and applied at the bottom of a gradient consisting of 0.5 ml 40% (w/v) sucrose, 1.5 ml 20% (w/v) sucrose and 1.5 ml 5% (w/v) sucrose, followed by centrifugation at 40 000 × g for 3 h in an SW55 rotor. Fractions were collected from the top of the gradient. For liposomes prepared with RITC-PE, the fluorescence of the fractions was measured in a Perkin-Elmer fluorimeter.

Binding assays for [^{125}I]EGF and [^{125}I]EGFR1: Liposomes prepared using 1 μg receptor protein were pelleted by centrifugation and resuspended in 50 μl TBS/0.1% BSA. [^{125}I]EGF (1.0 μCi, 27 ng) or [^{125}I]EGFR1 (0.7 μCi, 0.1 μg) were added and incubated for 1 h at room temperature. In control experiments, the liposomes were preincubated with a 100-fold excess of unlabelled EGF or EGFR1 for 30 min. The liposomes were floated up sucrose gradients as above. Fractions were collected from the top and their radioactivity measured in a γ-counter.

Treatment with proteases: Protease inhibitors were omitted from the last change of dialysis buffer and liposomes were incubated with 5 μg/ml protease in TBS for 1 h at room temperature. Protease inhibitors (30 mM benzamidine, 0.1 mM PMSF, 5 mM EDTA) were then added and after 30 min the liposomes were washed 3 times in TBS by centrifuging for 10 min at 100 000 × g in a Beckman Airfuge.

Immunoprecipitation of EGF receptor: Liposomes were lysed in 50 mM Tris, 150 mM NaCl, 5 mM EDTA, 1% NP40, pH 8.0, for 30 min at room temperature. 3 μg/ml of antibody EGFR1 were added and incubated for 1 h at room temperature. Immune complexes were precipitated using protein A-Sepharose and [^{125}I]EGF binding assays were done directly on the beads as described above for liposomes. The beads were washed 6 times in 50 mM Tris, 400 mM NaCl, 5 mM EDTA, 1% NP40, pH 8.0, and radioactivity measured in a γ-counter.

3. RESULTS AND DISCUSSION

3.1. *Reconstitution of the EGF receptor*

Octylglucoside was used for the isolation and reconstitution of the EGF receptor in liposomes because this detergent, having a high critical micelle concentration (25 mM), is rapidly and efficiently removed from protein and phospholipids by dialysis. The same method has been used successfully for the reconstitution of several other membrane proteins [10–14]. Mixing of purified receptor with phospholipids followed by removal of the detergent resulted in the formation of liposomes which were either pelleted by centrifugation at 100 000 × g for 1 h or were collected and separated from unincorporated materials by floating up a sucrose step gradient. To determine the position of the liposomes on the gradient a fluorescent derivative of PE was used in the reconstitution mixture. Free RITC-PE floated to the top of the gradient but a proportion of the fluorescent phospholipid banded at the 5–20% sucrose interface (fig.1). The association of EGF receptor with liposomes at this interface was determined by using biosynthetically labelled receptor, purified from cells incubated with [^{35}S]methionine,

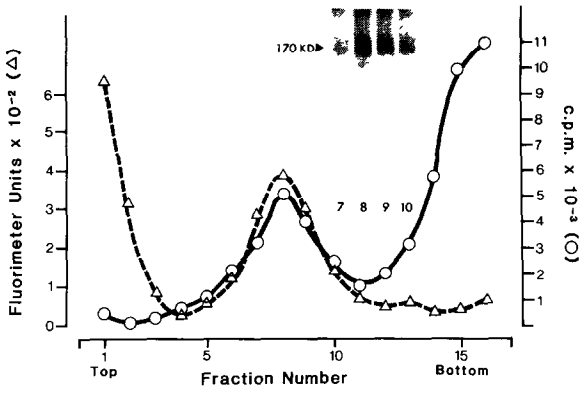

Fig.1. Density-gradient centrifugation of liposomes. Liposomes were prepared either with RITC-phosphatidylethanolamine (Δ) or with [^{35}S]methionine labelled EGF receptor (0.05 μCi) (○) as described in section 2. The fluorograph shows SDS-PAGE in 9% gel of fractions 7–10.

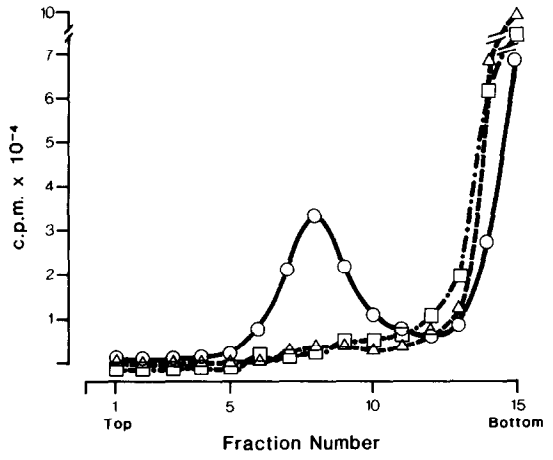

Fig.2. Binding of [^{125}I]EGF to reconstituted EGF receptors. Binding assays were done as described in section 2 on intact liposomes (○), liposomes pre-incubated with an excess of unlabelled EGF (Δ) and protease-treated liposomes (□).

and analysing fractions by scintillation counting and SDS-PAGE followed by fluorography (fig.1). Approx. 30% of the total amount of receptor was associated with the liposomes.

When material from the 5–20% interface was examined by electron microscopy using negative stain, typical vesicular structures were seen.

3.2. *Binding of labelled ligands*

The ability of the reconstituted receptor to bind specific ligands was tested using [^{125}I]EGF and [^{125}I]EGFR1, a monoclonal anti-EGF receptor antibody [15]. Unbound ligands were removed by density gradient centrifugation. The reconstituted receptor was able to bind both [^{125}I]EGF and [^{125}I]R1. Binding of both ligands was specific, because it was prevented when the liposomes were preincubated with an excess of unlabelled ligand (fig.2, data shown only for EGF). No binding was observed, under the same conditions, to liposomes prepared without EGF receptor.

3.3. *Effects of protease treatment*

EGF receptor could have been associated with the liposomes in 3 ways: inserted in the bilayer, trapped inside, or adsorbed to the external lipid leaflet. To distinguish between these possibilities, experiments using proteolytic digestion were performed. It was first shown that when liposomes were exposed to protease under isotonic, detergent-free conditions before testing for [^{125}I]EGF binding, more than 95% of the specific binding was lost (fig.2). However, when liposomes incorporating [^{35}S]methionine labelled EGF receptor were treated with proteases, about half of the radioactivity was lost (table 1), suggesting that a

Table 1

Effects of protease treatment on liposomes

(1) [^{35}S]EGF receptor liposomes	cpm at 5–20% interface
– Protease	9320
+ Protease	5270

(2) [^{125}I]EGF binding on immunoprecipitated receptor	cpm on protein A-Sepharose beads
– Protease	5391 ± 140
+ Protease	638 ± 28
– EGF receptor	347 ± 50

(1) Radioactivity associated with the liposomes before and after protease treatment: Liposomes were prepared with 0.03 μCi [^{35}S]EGF receptor and half were treated with proteases as described in section 2. (2) Liposomes, either untreated or protease treated were lysed and EGF receptors immunoprecipitated. [^{125}I]EGF binding was tested on the protein A-Sepharose beads. To estimate background binding, liposomes were prepared without EGF receptor and tested in the same way

part of the EGF receptor molecule, lacking the EGF-binding site, remained associated with the liposomes and was protected from proteolytic degradation. When protease-treated [^{35}S]EGF receptor bearing liposomes were analysed by SDS-PAGE, a single 92-kDa band appeared, whereas protease treatment in the presence of detergent resulted in much smaller fragments of the receptor (fig.3). Similar small fragments would have been produced if the receptor was adsorbed to the outside but did not span the lipid bilayer, whereas a band corresponding to intact receptor would have been seen if receptor was trapped inside the liposomes and protected from proteases.

Loss of EGF binding after protease treatment of intact liposomes does not necessarily mean that most of the reconstituted receptor molecules are 'correctly' inserted in the bilayer, i.e., with the EGF binding domain facing the medium, as in the cell membrane. To test whether insertion was random, liposomes, either intact or protease treated, were lysed with detergent and the EGF receptors immunoprecipitated with the monoclonal antibody EGFR1 and protein A-Sepharose. An [^{125}I]EGF binding assay was then done directly on the beads. It was found that protease treatment resulted in the loss of about 95% of [^{125}I]EGF binding sites (table 1). This result demonstrates that the majority of the EGF-binding sites are oriented to the outside of the liposomes and only a small proportion (< 5%) within the liposomes.

We believe that this reconstitution system will be important in the study of the EGF receptor molecule and its interactions with other cellular components. For this purpose we are developing a method for the efficient transfer of the reconstituted EGF receptors to receptor-negative cells.

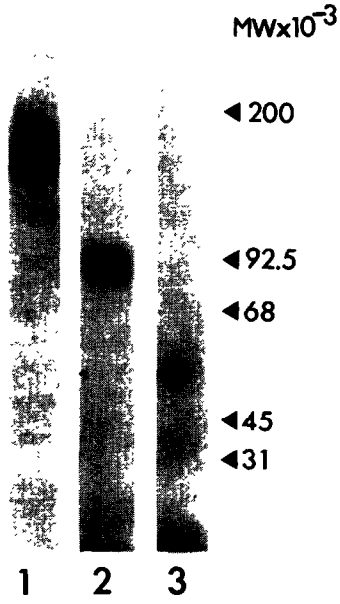

Fig.3. Effect of protease treatment on the reconstituted EGF receptor. Fluorograph of SDS-PAGE (5–15% gradient gel) of intact [^{35}S]EGF receptor liposomes (1), liposomes treated with proteases (2) and liposomes lysed in detergent and treated with proteases (3)

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