Biochimica et Biophysica Acta 939 (1988) 355–365
Elsevier

BBA 73951

Characterization of plasma membranes from A431 cells, isolated by self-generating Percoll gradient: a rapid isolation procedure to obtain plasma membranes with functional epidermal growth factor receptors

Bernard Payrastre [a], Monique Plantavid [a], Chantal Etievan [b], Gérard Ribbes [a], Claude Carratero [c], Hugues Chap [a] and Louis Douste-Blazy [a]

[a] *INSERM Unité 101, Biochimie des Lipides, Hôpital Purpan, Toulouse,* [b] *Laboratoires P. Fabre, Castres, and* [c] *Laboratoire de Biologie Médicale, Faculté de Médecine, Toulouse (France)*

(Received 2 October 1987)
(Revised manuscript received 24 December 1987)

Key words: Plasma membrane; Percoll gradient; Epidermal growth factor receptor; Electron microscopy; (A431 cell)

Plasma membranes have been isolated from the human epidermoid carcinoma cell line A431 by a rapid fractionation of lysate on Percoll density gradient at pH 9.6. Endoplasmic reticulum, lysosomes and mitochondria sedimented at the bottom of gradient whereas plasma membranes focused at low density, as shown with specific markers. Plasma membranes displayed a 4.5- and 4.4-fold enrichment in [^{3}H]concanavalin A and 5′-nucleotidase, respectively. This proteic fraction was further characterized by its lipid composition and phospholipid analysis. The cholesterol/phospholipid molar ratio was 0.45 in plasma membranes against 0.19 in lysate. Sphingomyelin increased from 7.5% of total phospholipids in lysate to 16.2% in plasma membranes, as well as phosphatidylserine which displayed a 1.5-fold enrichment in the plasma membrane fraction. This was at the expense of phosphatidylcholine (45.2% in lysate, against 35% in plasma membranes). Electron microscopy of the isolated material showed vesicles essentially free from endoplasmic reticulum and organelles. These plasma membranes retained the ability to bind ^{125}I-labelled epidermal growth factor (^{125}I-EGF) with a $K_d = 4.7$ nM and $B_{max} = 63$ pmol/mg protein. EGF binding resulted in a stimulation of the phosphorylation protein reaction in the presence of [γ-^{32}P]ATP and sodium dodecyl sulfate polyacrylamide gels of phosphorylated proteins indicated that the radioactivity of the major band of molecular weight 170 000 was clearly enhanced by EGF binding. These results indicate that the EGF receptor and its intrinsic protein kinase activity were preserved during our plasma membrane isolation procedure.

Introduction

The human epidermoid carcinoma cell line A431 is known to contain high concentration of membrane receptors for epidermal growth factor (EGF) to a level about 20–50-fold higher than normal cells [1–3]. For that reason, A431 cells are largely used as a biochemical model to precise interactions of EGF with its receptor and early events occurring after EGF-receptor complex formation. These include protein tyrosine phosphorylation [4], Ca^{2+} signal [5–7], as well as phosphoinositide metabolism [5,8–11]. However these studies also require the use of either permeabilized

Abbreviations: EGF, epidermal growth factor; DMEM, Dulbecco's modified Eagle's medium.

Correspondence: H. Chap, INSERM Unité 101, Biochimie des Lipides, Hôpital Purpan, 31059 Toulouse, France.

cells or isolated membranes. This is the case for investigations dealing with characterization of EGF receptor kinase [12–18] or with GTP-dependence of phospholipase C, as it was established in other cell systems [19–25].

Previous studies in our laboratory, dealing with several cell types including Krebs II ascite cells [26], neutrophils [27] and platelets [28,29], have achieved a successful separation of plasma membranes from intracellular membranes and organelles by centrifugation on Percoll gradient. In all instances, pH has been reported as a critical point of this one-step, rapid procedure. From these observations, we have used the same technical approach to purify plasma membranes from A431 cells. We report here biochemical and electron microscopy characterization of this fraction. Furthermore, the best known functions of EGF receptor such as EGF binding and EGF receptor autophosphorylation by the intrinsic tyrosine-specific protein kinase activity have been investigated. Our results allow us to propose such a procedure for further studies on metabolic events evoked by EGF binding.

Materials and Methods

Materials

Percoll (poly(vinyl pyrollidone)-coated silica particles) was from Pharmacia, Uppsala, Sweden. Dulbecco's modified Eagle's medium (DMEM) and fetal calf serum were from Gibco, Paiskey, Renfrewshire, Scotland (U.K.). Epidermal growth factor and all other chemicals were obtained from Sigma, St Louis, MO, U.S.A. [^{3}H]Concanavalin A (60 Ci/mol), [^{3}H]AMP (15 Ci/mmol), [γ-^{32}P]ATP (3000 Ci/mmol), ^{125}I-epidermal growth factor (100 μCi/μg) were purchased from Amersham International, Amersham, U.K.

Cell culture

A431 cells (a gift from Dr. Lapière, Liège, Belgium) were plated in culture dishes (150 mm × 25 mm) using Dulbecco's modified Eagle's medium (DMEM) containing 5% (v/v) fetal calf serum, 100 μg/ml penicillin and 100 μg/ml streptomycin, in a gas phase of 5% (v/v) CO_2 in air. Postconfluent cells were scraped into NaCl (9 g/l) and sedimented by centrifugation at 200 × g for 10 min. The pellet was then washed in 40 vol. of an isotonic buffer containing 25 mM Tris, 125 mM KCl, 5 mM $MgCl_2$ (pH 7.4). Finally, cells were suspended in the same buffer to a final cell suspension of 30% (v/v). All the washing procedure was performed at room temperature.

Cell surface labelling by tritiated concanavalin A

20 ml of the 30% cell suspension were incubated with 2 μCi of [^{3}H]concanavalin A for 10 min at 20 °C, under gentle magnetic stirring. Cell pellet was washed twice by 40 vol. of the same buffer and resuspended in 10 ml of a lysis buffer containing 25 mM Tris, 125 mM KCl, 5 mM $MgCl_2$, 1 mM ATP (pH 9.6).

Subcellular fractionation and plasma membrane isolation

Cell disruption was performed at 4 °C by the nitrogen cavitation technique in a Kontes pressure homogenizer (Kontes, Vineland, NJ, U.S.A.) as previously described [26], except that cells were submitted to 60 bars for only 5 min. The following procedure was then essentially the same as described [26]. Briefly, the pH of the 1000 × g supernatant was adjusted to 9.6 with a few drops of 0.25 M NaOH and 4 ml were added to a previously prepared mixture of 11 ml Percoll, 2.2 ml distilled water and 4.8 ml 400 mM KCl, 20 mM $MgCl_2$, 400 mM Tris (pH 9.6). At this step, pH was carefully adjusted to 9.6 again. Subcellular fractionation was then achieved by centrifugation at 160 000 × g for 10 min at the plateau in a Beckman 60 Ti rotor. Then, 2-ml fractions were harvested from the top of the Percoll gradient. [^{3}H]Concanavalin A radioactivity was counted directly from 100 μl of each fraction, whereas Percoll was eliminated before protein and enzymatic measurements. This was achieved by centrifugation at 130 000 × g for 30 min after a 3-fold dilution of each fraction with 50 mM Tris (pH 7.4). Percoll beads stuck at the bottom of the tube whereas proteins layed on the surface of the Percoll cushion. They were collected in 50 mM Tris (pH 7.4) and sonicated 2 × 10 seconds using an MSE sonicator at maximum output for homogenization. In current plasma membrane preparations, fractions 4 and 5 of the Percoll gradient corresponding to the visible upper band were pooled,

diluted with 50 mM Tris (pH 7.4) and submitted to a first centrifugation at 130000 × *g* for 30 min to obtain Percoll stuck at the bottom of the tube. Then the tube was submitted to a slight vortex agitation so that membranes slide away from above Percoll pellet. Supernatant was poured into another tube and a second run at 160000 × *g* for 35 min was performed. Membrane pellet was then harvested.

Enzymatic determination

Cytosolic lactate dehydrogenase (EC 1.1.1.27) was assayed as described in Ref. 30 using the method of Wroblewski and La Due [31]. 5′-Nucleotidase (EC 3.1.3.5), NADH dehydrogenase (EC 1.6.99.3), also called NADH diaphorase, *N*-acetyl-β-D-glucosaminidase (EC 3.2.1.30) and succinate–cytochrome-*c* reductase (EC 1.3.99.1) were measured exactly as described by Record et al. [26].

Binding of [^{125}I]-EGF to plasma membranes

The binding reaction was carried out at 24°C for 30 min with 8 μg protein according to the protocol described by Carpenter et al. [12] except that sample was diluted at the end of the incubation with 3 ml of ice-cold 20 mM Hepes buffer (pH 7.4) before filtering on Sartorius cellulose acetate filters (SM 11107). Each filter was then washed three times with 3 ml of the same cold buffer, dried and determined for radioactivity.

EGF-stimulated protein phosphorylation

Plasma membranes (10–20 μg protein) were phosphorylated by [γ-^{32}P]ATP (0.5 μCi, 15 μM) essentially as described by Carpenter et al. [12], for 1 min at 0°C in the absence or in the presence of EGF at the concentrations indicated. The reaction was terminated by addition of 50 μl cold 20% trichloroacetic acid containing 0.02 M sodium pyrophosphate. Assays were then immediately filtered on glass fiber filters (GFC Whatman). These were washed with 5 × 3 ml 10% trichloroacetic acid/0.01 M sodium pyrophosphate followed by 2 × 3 ml ethanol and 5 ml ether and the radioactivity was measured.

When ^{32}P-labelled proteins were submitted to polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate (SDS-PAGE), the phosphorylation reaction was blocked with the electrophoresis buffer containing 6.6% SDS, 0.6 M mercaptoethanol, 15 mM EDTA, 60% glycerol and 0.05% Bromophenol blue (pH 8.6). Assays were heated in boiling water for 2 min and SDS-PAGE was carried out as described by Laemmli [32] using a 7% polyacrylamide gel. Gels were run at 200 V in a Hoeffer apparatus (vertical electrophoresis SE600). Commercial molecular weight standards were used for calibration of gels. After migration, proteins were stained with Coomassie brilliant blue solution, destained by repeated washings in acetic acid/methanol/water (10:10:80, v/v). Autoradiography was achieved after drying gels using X-ray film, at −70°C.

Electron microscopy of plasma membrane fraction

Plasma membrane pellets obtained by 160000 × *g* for 35 min were placed into an Eppendorf microfuge tube and further centrifuged for 10 min. Pellet was fixed in 2% glutaraldehyde for 30 min and postfixed with 2% (w/v) osmium tetroxide in 0.2 M phosphate (pH 7.2) for 1 h. Specimens were dehydrated with graded concentrations of ethanol and propylene oxyde and embedded in Epon. Thin sections were cut on a Reichert OM U2 ultramicrotome, mounted on grids and stained with uranyl acetate and lead citrate. They were viewed in a Hitachi H300 electron microscope.

Lipid analysis

Lipids were extracted according to Bligh and Dyer [33]. Total phospholipid content was estimated by phosphorus measurement according to Bôttcher [34]. Their separation was performed by two-dimensional thin-layer chromatography on silicagel-coated plates (Merck) using chloroform/methanol/water/acetic acid (75:45:6:12, v/v) as the first solvent [35] and chloroform/methanol/0.9% saline/acetic acid (100:15:4:16) as the second solvent [36]. The different spots were scraped off and analysed for their phosphorus content. Cholesterol was determined by gas-liquid chromatography as previously described [29].

Other analytical methods

Protein was determined by the method of Lowry et al. [37] using bovine serum albumin as a stan-

dard. ^{3}H and ^{32}P radioactivity was counted with a Kontron Intertechnique liquid scintillation spectrometer (type SL4000) equipped with automatic quenching correction, using Instagel (Packard) as scintillation fluid. ^{125}I radioactivity was measured with a Packard Autogamma 5780 spectrometer.

Results

Cell disruption and low-speed centrifugation of the cell lysate

Measurement of soluble lactate dehydrogenase activity allowed us to verify the efficiency of the cavitation procedure under our experimental conditions, since the equilibration time under 60 atm of N_2 was reduced from 20 min [26] to 5 min. Over 90% of total lactate dehydrogenase activity was found in the supernatant obtained after centrifugation at $1000 \times g$ for 10 min to sediment nuclei and intact cells. About 70% of [^{3}H]concanavalin A and 80% of 5′-nucleotidase, chosen as markers of plasma membranes [27] were recovered in the low-speed supernatant, which contained 60% of intracellular membranes, as indicated by determination of NADH dehydrogenase activity. Therefore further plasma membrane separations were performed from the low-speed supernatant.

Subcellular fractionation on Percoll gradient

Because previous studies have underlined the effect of pH on the apparent density of intracellular membranes and organelles [26–29], we first investigated the influence of gradient pH on the separation of plasma membranes from endoplasmic reticulum, mitochondria and lysosomes (Fig. 1). Increase of pH from 9.06 to 9.6 improved drastically the separation between plasma membranes and the other intracellular elements. [^{3}H]Concanavalin A always focused in fraction 4 (numbered from the top of the gradient), whereas some population of mitochondria (as determined by succinate–cytochrome-*c* reductase activity), lysosomes (with *N*-acetylglucosaminidase as marker) and endoplasmic reticulum shifted from the low densities at the top of the tubes to the bottom of the gradient. An intermediate pH of 9.1 was sufficient to well separate plasma membranes from mitochondria, but higher pH values were necessary to recover the whole population of

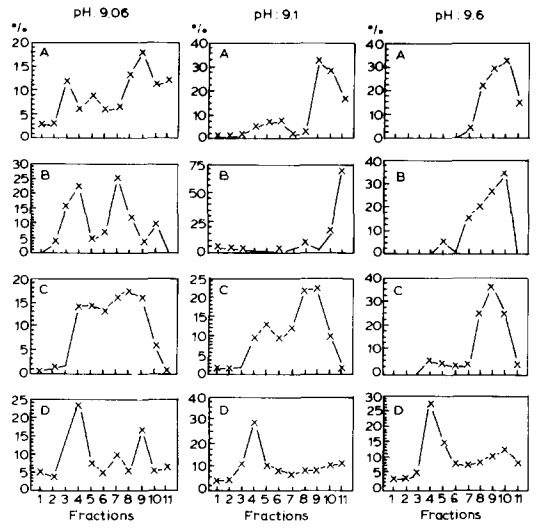

Fig. 1. Influence of pH on the separation of A431 plasma membrane from intracellular membranes and organelles on Percoll gradients. Three gradients were run at pH 9.06, 9.1 and 9.6, respectively. Data correspond for enzymes to percentages of total activity present in the whole gradient and for [^{3}H]concanavalin A to percentages of total radioactivity. (A) NADH dehydrogenase; (B) succinate–cytochrome-*c* reductase; (C) *N*-acetyl-β-D-glucosaminidase; (D) [^{3}H]concanavalin.

lysosomes and endoplasmic reticulum vesicles in the high-density fractions. So, pH 9.6 was routinely used in all further fractionations. As illustrated in Fig. 2, 5′-nucleotidase displayed the same profile as [^{3}H]concanavalin A. The two markers peaked in fractions 4 and 5 which contained 40% of total

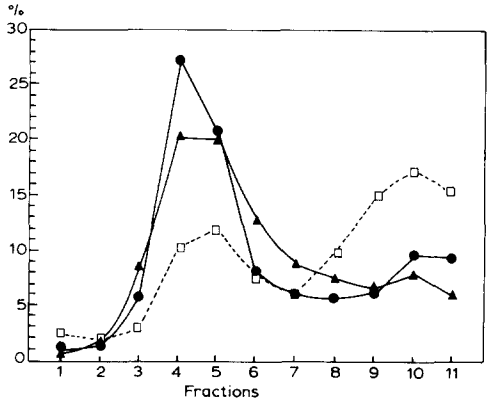

Fig. 2. Similar localization of [^{3}H]concanavalin A and 5′-nucleotidase in Percoll gradient achieved at pH 9.6. Results correspond to a typical fractionation and are expressed as in Fig. 1. ●———●, [^{3}H]concanavalin A; ▲———▲, 5′-nucleotidase; □-----□, protein percentages in each fraction.

TABLE I

SPECIFIC ACTIVITIES OF [^{3}H]CONCANAVALIN AND ENZYME MARKERS IN CELL LYSATE AND PLASMA MEMBRANE PREPARATION

Results are means ± S.E. from $n = 4$–6 experiments. n.d., not detectable.

Markers	Cell lysate	Plasma membranes	Enrichment ratio
[^{3}H]Concanavalin A (dpm/mg protein)	41005 ± 13286 ($n = 6$)	185019 ± 66394 ($n = 6$)	4.5
5′-Nucleotidase (nmol/h per mg protein)	117 ± 15 ($n = 5$)	517 ± 100 ($n = 5$)	4.4
NADH-dehydrogenase (nmol/min per mg protein)	510 ± 92 ($n = 5$)	194 ± 66 ($n = 5$)	0.38
Glucosaminidase (nmol/h per mg protein)	88 ± 11 ($n = 4$)	38 ± 16 ($n = 4$)	0.43
Succinate–cytochrome-*c* reductase (nmol/min per mg protein)	3.9 ± 1.5 ($n = 4$)	n.d.	–
Total proteins (mg)	35 ± 6.2 ($n = 6$)	1.36 ± 0.32 ($n = 6$)	

5′-nucleotidase and 47% of total [^{3}H]concanavalin A from the 1000 × *g* supernatant. However, fraction 5 always contained relatively more 5′-nucleotidase than [^{3}H]concanavalin. Fractions 4 and 5 represented 22% of total proteins across gradient from the 1000 × *g* supernatant and corresponded to the low-density band visible at the top of the gradient. They were routinely pooled together for plasma membrane preparation. Specific activities of various markers and protein content of plasma membrane preparations are reported in Table I and compared to values obtained for the corresponding cell lysates. Enrichment ratio for 5′-nucleotidase and for [^{3}H]concanavalin A, the two markers of plasma membranes are similar, 4.4- and 4.5-fold, respectively. The plasma fraction was only slightly contaminated by internal membranes and lysosomes, whereas the activity of the mitochondrial marker was not detectable. Protein content represented 3.8 ± 0.6% ($n = 6$) of the total protein from the cell lysate, whereas the yield of the two plasma membrane markers ([^{3}H]concanavalin A and 5′-nucleotidase) indicated that about 17–20% of total plasma membrane was recovered in a purified form using this procedure.

Lipid analysis

As shown in Table II, cholesterol was enriched 7.7-fold in plasma membranes over whole cells, and 3.3-fold for phospholipids. As a consequence, the cholesterol/phospholipid molar ratio was increased from 0.19 to 0.45 between cell lysate and plasma membrane. Table III describes the phospholipid composition of the plasma membrane fraction comparatively to cell lysate. Plasma membranes displayed a 2-fold enrichment in sphingomyelin. There was also an increase in

TABLE II

LIPID COMPOSITION OF CELL LYSATE AND PLASMA MEMBRANES

Results represent means ± S.E. of five different preparations.

	Cell lysate	Plasma membranes
Phospholipids (nmol/mg protein)	305 ± 38	1017 ± 226
Cholesterol (nmol/mg protein)	59 ± 6	455 ± 44
Cholesterol/phospholipids (molar ratio)	0.19	0.45

TABLE III

PHOSPHOLIPID COMPOSITION OF CELL LYSATE AND PLASMA MEMBRANES

Results are molar percentages of total in each class of phospholipids and represent means ± S.E. of four or five different preparations.

	Total cell	Plasma membranes
Sphingomyelin	7.5 ± 0.7	16.2 ± 0.8
Phosphatidylcholine	45.2 ± 0.7	35.0 ± 1.2
Phosphatidylserine	8.5 ± 0.7	13.0 ± 0.8
Phosphatidylinositol	7.8 ± 1	6.6 ± 0.9
Phosphatidylethanolamine	31 ± 0.7	29.1 ± 1.2

phosphatidylserine, whereas phosphatidylcholine and phosphatidylinositol were less abundant in plasma membranes than in whole cells.

Electron microscopy of A431 plasma membrane preparation

Electron micrographs of thin-sectionned purified plasma membranes (Figs. 3A and 3B) showed a population of membrane vesicles of different sizes. Some of them gave a rough aspect owing to the entrapment of hyaloplasmic material. Membrane sheets were also detected in preparation viewed in Fig. 3B. No contamination by nuclei or mitochondria could be detected. Also, attached

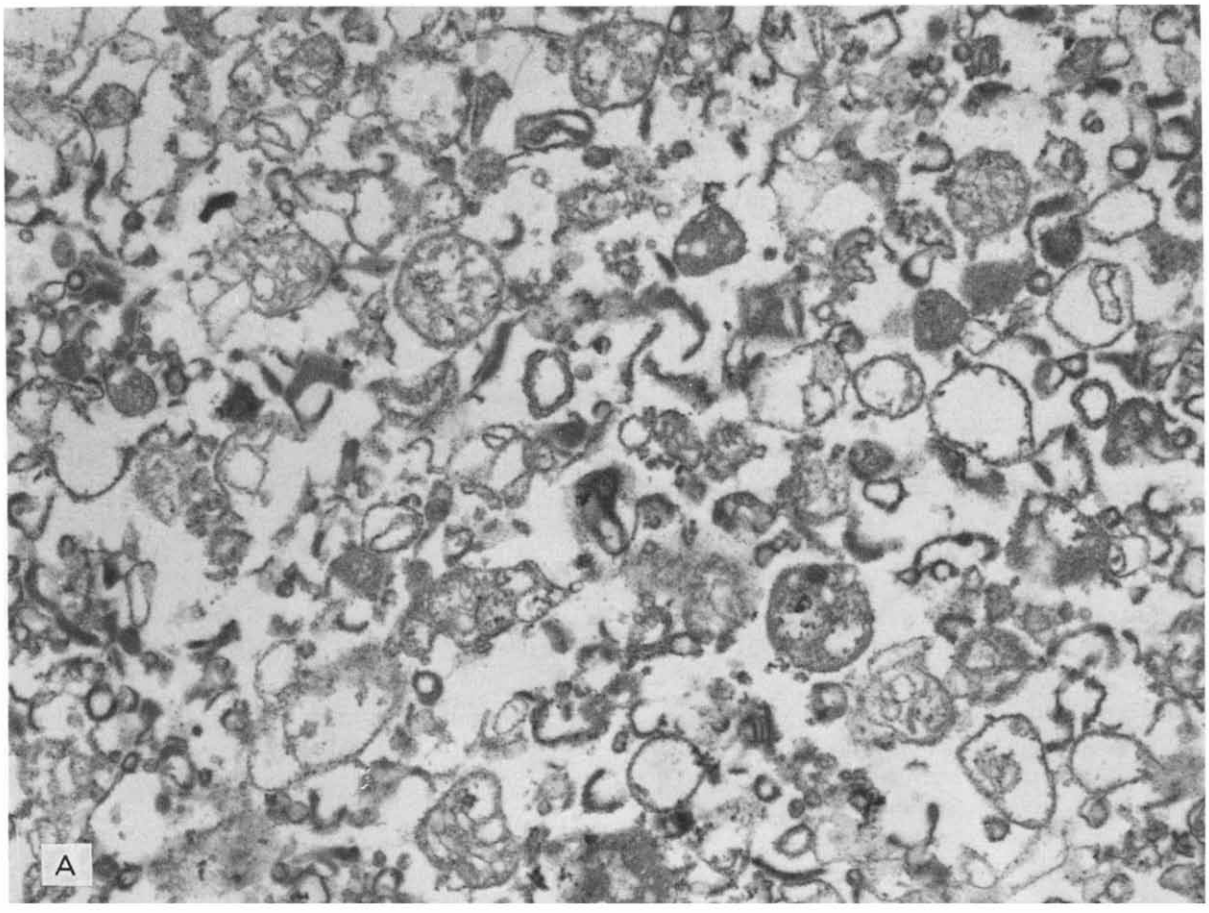

Fig. 3. Electron micrographs of A431 plasma membrane fraction (A: ×15000, B: ×40000) prepared as described under Materials and Methods.

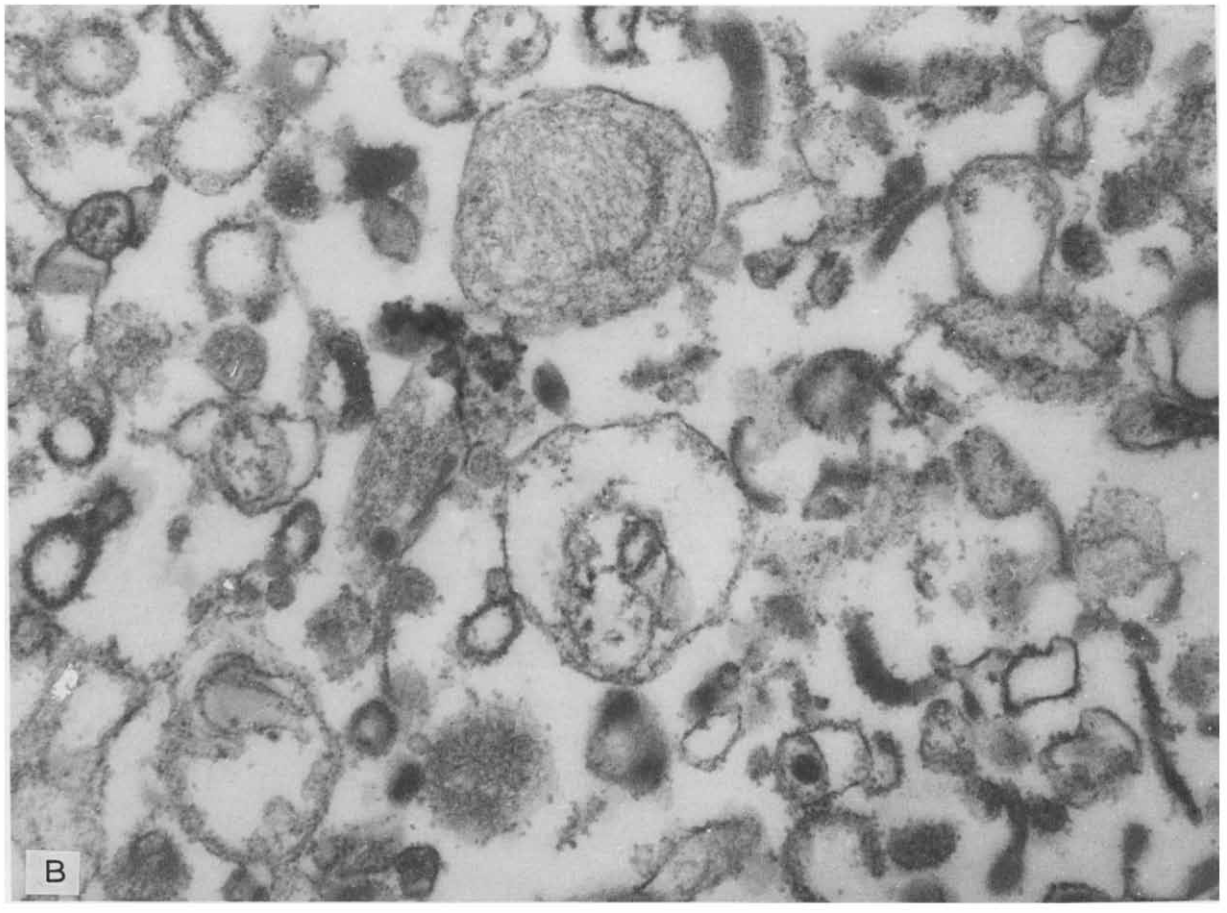

Fig. 3 (continued).

ribosomes, which were clearly visible in the high-density fractions from the gradient (not shown), were not observed in the plasma membrane fraction. Some more electron dense particles of smaller size and with occasionnally elongated shape were also present all over the preparation. Since no gross contamination by lysosomes was evident from biochemical markers (see Fig. 1 and Table I), these structures were interpreted as membrane sheets with a pseudopod-like aspect. Percoll beads (about 17 nm diameter) were hardly or not visible.

^{125}I-EGF binding to plasma membranes

The specific binding of ^{125}I-EGF to the purified plasma membranes is described in Fig. 4. In the presence of a 100-fold molar excess of unlabelled EGF, non-specific binding did not exceed 10% of total binding. Scatchard plot analysis (inset) revealed the presence of a single class of binding sites (B_m 63 pmol/mg protein and K_d 4.7 nM).

Protein phosphorylation of A431 purified membranes. Effects of EGF

Total protein kinase activity of the plasma membrane preparation was assayed with [γ-^{32}P]ATP as substrate, in the absence or in the presence of increasing concentrations of EGF. Despite a high background of radioactivity, addition of 120 nM EGF stimulated ^{32}P incorporation into acid precipitable protein by 24% ($P < 0.01$). However, the stimulating effect of EGF was more evident when labelled proteins were further analyzed by SDS-PAGE and autoradiography. As

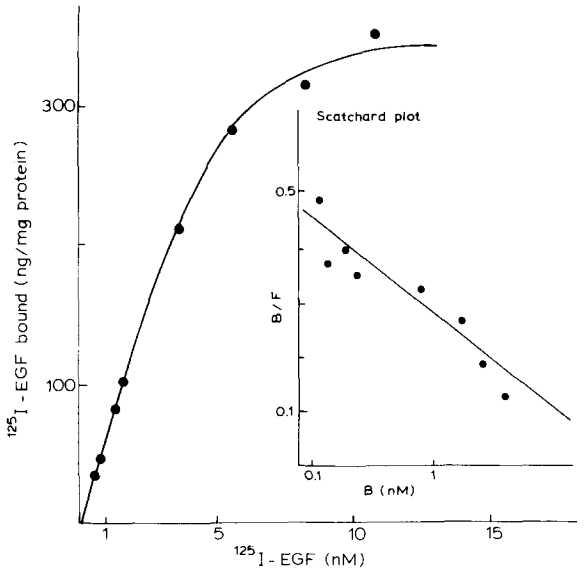

Fig. 4. ^{125}I-EGF binding to A431 plasma membrane preparation. Effect of increasing EGF concentration. Plasma membranes (8 μg) were incubated with various concentrations of ^{125}I-EGF (220000 dpm/ng) at 24° C for 30 min as described under Materials and Methods. At each concentration, duplicate tubes for total and non specific binding were assayed. Points represent specific binding obtained with a typical membrane preparation and inset corresponds to Scatchard analysis of binding data.

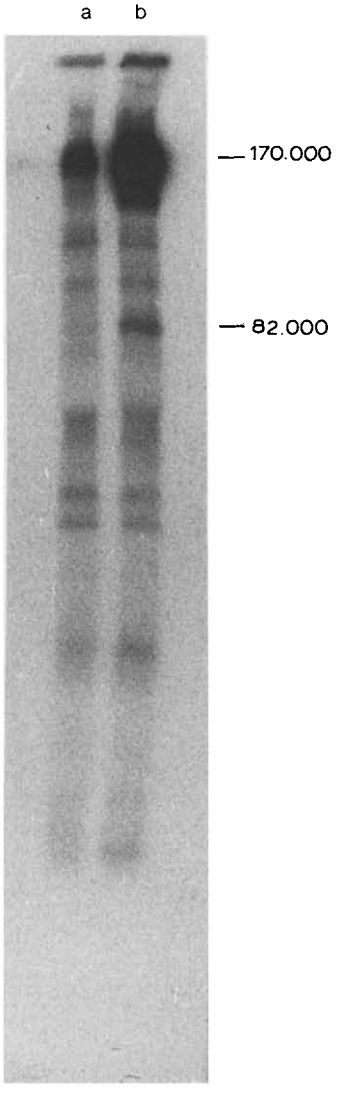

Fig. 5. EGF receptor autophosphorylation. Autoradiography of an SDS-electrophoresis gel of plasma membrane proteins following incubation of A431 membrane fraction with [γ-^{32}P]ATP. A431 plasma membranes (20 μg proteins) were phosphorylated at 4° C for 1 min with 0.5 μCi [γ-^{32}P]ATP and in the absence (line a) or in the presence (line b) of 120 nM EGF, as described under Materials and Methods.

shown in Fig. 5, the major phosphorylated band whose labelling was enhanced by EGF was detected at an apparent M_r of 170000. This corresponds to the value determined previously for EGF receptor [16]. Another radioactivity band was also observed just below and probably corresponds to the 150 kDa proteolytic fragment of the receptor [16,18]. An additional band with a M_r of 82000 was also phosphorylated in an EGF-dependent manner and might correspond to the 81 kDa protein previously described by others [38,39]. However, no clear phosphorylation was observed in the M_r 36000 region.

Discussion

This paper deals with the description of a new isolation procedure of A431 plasma membranes. Using this acellular model, extensive studies have already shown that EGF receptor was able to autophosphorylate on tyrosine residues and that EGF binding resulted in an enhancement of this intrinsic protein kinase activity [15,16]. However, other biochemical events may also contribute to the action of EGF on A431 cells, since phospholipase C could be involved in EGF transduction in these cells [5,8,11]. Further studies on isolated plasma membranes are still necessary to better understand the mechanism of these activa-

tions and to investigate possible pathways such as process mediated by guanine nucleotide binding proteins. Till now, plasma membranes from A431 cells were prepared in most cases by the method of Thom et al. [40] adapted to this cell line by Carpenter et al. [12]. The procedure includes a hypoosmotic lysis followed by differential centrifugation and centrifugation on a sucrose barrier. Since another more rapid technical approach was extensively used in our laboratory to purify plasma membranes from several cell types [26–29], we have adapted this method to A431 cells. The new procedure requires less than 90 min.

Two technical points are essential in our method, as previously discussed by Record et al. [26]: (1) the presence of ATP plus Mg^{2+} in the lysis buffer, which prevents polymerisation of cytoskeletal protein leading to poor yields in plasma membrane material and (2) the pH of Percoll gradient, which provokes variations in the apparent density of internal membranes and organelles. An alkaline pH (9.6) was necessary for a complete separation of plasma membranes from intracellular membranes and lysosomes, whereas mitochondria already focused in the high density band at a less alkaline pH.

Various data in our study support the idea that the fraction isolated from the upper band of Percoll gradient at pH 9.6 indeed corresponds to plasma membranes. At first, [^{3}H]concanavalin A which, at the low concentrations used, can be considered as a good probe of external cell surface [26] as well as 5′-nucleotidase display an appreciable enrichment ratio. On the opposite, markers of endoplasmic reticulum and organelles are significantly reduced or even absent in our preparation.

Further lipid analysis confirms these results. The cholesterol and phospholipid contents measured in cell lysate and membrane preparation are in good agreement with previous literature data [27,29]. A surprising observation was the higher enrichment in cholesterol content of plasma membrane (7.7-fold), compared to [^{3}H]concanavalin A and 5′-nucleotidase, which appeared to be enriched only 4.5-fold. This might indicate some loss of the two latter markers during the purification procedure (including detachment of the lectin as well as inactivation of the enzyme). This suggests that the enrichment of the plasma membrane fraction might have been higher than anticipated from data on the protein markers. In the same way, the comparison of phospholipid composition between whole cells and plasma membranes which displays a 2-fold enrichment in sphingomyelin and a decrease in phosphatidylcholine content provides further evidence for a high purity of these membranes. Electron microscopic examination of the purified membranes allowed to visualize their vesiculation state and the absence of contamination by other cellular structure in the preparation. This also revealed a large amount of hyaloplasmic material remaining entraped inside the vesicles, resulting in a rather rough aspect as well as in the presence of some electron dense structures. Although plasma membranes prepared according to Thom et al. [40] displayed a different aspect [12,40], a rougher aspect was also recently reported by Lin et al. [41]. To date, it is difficult to understand the reasons for such morphological differences.

Concerning A431 cells, an essential aim is to preserve a functional EGF receptor. Membranes obtained in this study retained the ability to bind specifically ^{125}I-EGF. The affinity for EGF observed in the present study ($4.7 \cdot 10^{-9}$ M) is in the same range of magnitude as that reported for other plasma membrane preparations from A431 cells [12,16,41]. These probably correspond to the major form of EGF receptors present in A431 cells, since only a minute proportion (0.1–0.2%) of receptors with a 100-fold higher affinity have been reported in intact cells [42] but never in isolated membranes.

EGF receptor (M_r 170000) is a transmembrane protein which possesses in its internal domain a tyrosine protein kinase activity stimulated by EGF binding. The major protein phosphorylated by this kinase appears to be the EGF receptor itself [18]. In this study, when plasma membrane fraction was phosphorylated in the presence of [γ-^{32}P]ATP, EGF addition enhanced labelling of a major band with a M_r of 170000, supporting the view that EGF receptor functions were preserved during the isolation procedure. Beside the 81 kDa band previously described [38,39], which does not seem to correspond to phosphatidylinositol kinase detected in other cells [43], no significant phosphorylation of lipocortin (M_r 36000) could be observed. This is probably due to lipocortin elimination

during the washing procedure, since lipocortin binds to A431 plasma membrane in the presence of calcium [17].

In conclusion, the procedure described herein may represent a convenient method to prepare more rapidly than previously and with a suitable purity, plasma membranes from A431 cells containing a still functional EGF receptor. During the preparation of this manuscript, another rapid technique to isolate plasma membranes from this cell line was described by Lin et al. [41]. In that case, the purification procedure is based on the aggregation of intracellular membranes by high Ca^{2+} concentrations (up to 10 mM). The two procedures can be achieved within the same time (roughly 90 min), they apparently lead to comparable degrees of enrichment (around 5-fold for 5′-nucleotidase), although contamination by endoplasmic reticulum and lysosomes appeared somewhat higher in the method of Lin et al. [41]. However, the same marker enzymes were not used throughout. Further studies will be necessary to determine which of the conditions used in both procedures (alkaline pH or high calcium concentration) could be deleterious to some components of the plasma membrane. Apparently, this does not seem to be the case for EGF receptor and several works in our laboratory dealing with lipid studies [44,45] on plasma membranes from platelets isolated with a comparable preparative method including an alkaline pH step have shown that monoacylglycerol, phosphatidylinositol and phosphatidylinositol-4-phosphate kinases keep a full activity. Further studies on enzymes of lipid metabolism are now in progress on A431 plasma membranes isolated by the technique described in this paper.

Acknowledgements

This work was supported by Association pour la Recherche sur le Cancer (Villejuif, France). Thanks are due to Miss C. Cathala, Mr. C. Vieu and Miss L. Vrancken for technical assistance, to Dr. M. Record for helpful discussion and Mrs. Y. Jonquière for correcting the English manuscript.

References

1 Haigler, H.T., Ash, J.F., Singer, S.J. and Cohen, S. (1978) Proc. Natl. Acad. Sci. USA 75, 3317–3321.
2 Carpenter, G. and Cohen, S. (1979) Annu. Rev. Biochem. 48, 193–216.
3 Carpenter, G. and Zendegui, J.G. (1986) Exp. Cell Res. 164, 1–10.
4 Sawyer, S.T. and Cohen, S. (1985) J. Biol. Chem. 260, 8233–8236.
5 Sawyer, S.T. and Cohen, S. (1981) Biochemistry 20, 6280–6286.
6 Moolenaar, W.H., Aerts, R.J., Tertoolen, L.G.J. and De Laat, S.W. (1986) J. Biol. Chem. 261, 279–284.
7 Macara, I.G. (1986) J. Biol. Chem. 261, 9321–9327.
8 Smith, K.B., Losonczy, I., Sahai, A., Pannerselvam, M., Fehnel, P. and Salomon, D.S. (1983) J. Cell Physiol. 117, 91–100.
9 Pike, L.J. and Eakes, A.T. (1987) J. Biol. Chem. 262, 1644–1651.
10 Wahl, M.I., Sweatt, J.D. and Carpenter, G. (1987) Biochem. Biophys. Res. Commun. 142, 688–695.
11 Hepler, J.R., Nakahata, N., Lovenberg, T.W., Diguiseppi, J., Herman, B., Earp, H.S. and Harden, T.K. (1987) J. Biol. Chem. 262, 2951–2956.
12 Carpenter, G., King, L.J. and Cohen, S. (1979) J. Biol. Chem. 254, 4884–4891.
13 Cohen, S., Carpenter, G. and King, L. (1980) J. Biol. Chem. 255, 4834–4842.
14 King, L., Jr., Carpenter, G. and Cohen, S. (1980) Biochemistry 19, 1524–1528.
15 Ushiro, H. and Cohen, S. (1980) J. Biol. Chem. 255, 8363–8365.
16 Cohen, S., Ushiro, H., Stoscheck, C. and Chinkers, M. (1982) J. Biol. Chem. 257, 1523–1531.
17 Fava, R.A. and Cohen, S. (1984) J. Biol. Chem. 259, 2636–2645.
18 Cohen, S. (1983) Methods Enzymol. 99, 379–387.
19 Haslam, R.J. and Davidson, M.M.L. (1984) Nature (London) 312, 315–321.
20 Martin, T.F.J., Lucas, D.O., Bajjalieh, S.M. and Kowalchyk, J.A. (1986) J. Biol. Chem. 261, 2918–2927.
21 Merritt, J., Taylor, C.W., Rubin, R.P. and Putney, J.W. (1986) Biochem. J. 236, 337–343.
22 Gilman, A.G. (1984) Cell 36, 577–579.
23 Cockcroft, S. and Gomperts, B.D. (1985) Nature 314, 534–536.
24 Litosch, I., Wallis, C. and Fain, J.N. (1985) J. Biol. Chem. 260, 5464–5471.
25 Smith, C.D., Lane, B.C., Kusaka, I., Verghese, M.N. and Snyderman, R. (1985) J. Biol. Chem. 260, 5875–5878.
26 Record, M., Bès, J.C., Chap, H. and Douste-Blazy, L. (1982) Biochim. Biophys. Acta 688, 57–65.
27 Record, M., Laharrague, P., Fillola, G., Thomas, J., Ribbes, G., Fonta, G., Chap, H., Corberand, J. and Douste-Blazy, L. (1985) Biochim. Biophys. Acta 819, 1–9.
28 Perret, B., Chap, H.J. and Douste-Blazy, L. (1979) Biochim. Biophys. Acta 556, 434–446.

29 Fauvel, J., Chap, H., Roques, V., Lévy-Tolédano, S. and Douste-Blazy, L. (1986) Biochim. Biophys. Acta 856, 155–164.
30 Chap, H.J., Zwaal, R.F.A. and Van Deenen, L.L.M. (1977) Biochim. Biophys. Acta 467, 146–164.
31 Wroblewski, F. and La Due, J.S. (1955) Proc. Soc. Exp. Biol. 90, 210–215.
32 Laemmli, U.K. (1970) Nature (London) 227, 680–685.
33 Bligh, E.G. and Dyer, W.J. (1959) Can. J. Biochem. Physiol. 37, 911–918.
34 Bôttcher, C.J.F., Van Gent, C.M. and Pries, C. (1961) Anal. Chim. Acta 24, 203–204.
35 Skipsky, V.P., Peterson, R.F. and Barclay, M. (1964) Biochem. J. 90, 374–378.
36 Anderson, R.E., Maude, M.B. and Feldman, G.L. (1969) Biochim. Biophys. Acta 187, 345–353.
37 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
38 Carpenter, G., King, L. and Cohen, S. (1978) Nature 276, 409–410.
39 Gould, K.L., Cooper, J.A., Bretscher, A. and Hunter, T. (1986) J. Cell. Biol. 102, 660–669
40 Thom, D., Powell, A.J., Lloyd, C.W. and Rees, D.A. (1977) Biochem. J. 168, 187–194.
41 Lin, P.H., Selinfreund, R., Wakshull, E. and Wharton, W. (1987) Biochemistry 26, 731–736.
42 Kawamoto, T., Sato, J.D., Le, A., Polikoff, J., Sato, G.H. and Mendelsohn, J. (1983) Proc. Natl. Acad. Sci. USA 80, 1337–1341.
43 Kaplan, D.R., Whitman, M., Schaffhausen, B., Pallas, D.C., White, M., Cantley, L. and Roberts, T.M. (1987) Cell 50, 1021–1029.
44 Mauco, G., Fauvel, J., Chap, H. and Douste-Blazy, L. (1984) Biochim. Biophys. Acta 796, 169–177.
45 Mauco, G., Dajeans, P., Chap, H. and Douste-Blazy, L. (1987) Biochem. J. 244, 757–761.