(poly)Phosphoinositide phosphorylation is a marker for plasma membrane in Friend erythroleukaemic cells

André J. Rawyler, Ben Roelofsen, Karel W.A. Wirtz and Jos A.F. Op den Kamp

Biochemisch Laboratorium, Rijksuniversiteit Utrecht, Transitorium III, Padualaan 8, NL 3584 CH Utrecht, The Netherlands

Received 14 September 1982

Upon subcellular fractionation of (murine) Friend erythroleukaemic cells (FELCs), purified plasma membranes were identified by their high enrichment in specific marker enzymes and typical plasma membrane lipids. When FELCs were incubated for short periods with $^{32}P_i$ before cell fractionation, the lipid-bound radioactivity was almost exclusively present in phosphatidylinositol-4-phosphate (DPI) and phosphatidylinositol-4,5-*bis*phosphate (TPI), and its distribution closely matched that of the plasma membrane markers. In addition, purified plasma membranes actively incorporated ^{32}P from [γ-^{32}P]ATP into polyphosphoinositides, and the specific activities of the involved kinases were again mostly enriched in the plasma membrane fraction.

Friend erythroleukaemic cells *Cell fractionation* *Marker enzymes* *Plasma membrane, labeling* *(poly)Phosphoinositide phosphorylation*

1. INTRODUCTION

During our attempts to purify the plasma membrane of Friend erythroleukaemic cells (FELCs), specific labeling of the outer surface of the cells was required in order to estimate recovery of plasma membrane material during the purification. However, most of the attempts failed because of the high reactivity of the FELCs towards exogenously added labels resulting either in cell lysis or in label internalization. Therefore, we tried to achieve labeling via a naturally occurring, membrane-bound metabolic event and one of the most obvious candidates turned out to be the (poly)-phosphoinositide phosphorylation. This process has been studied extensively [1–11] and evidence has been presented that it is located preferentially in the plasma membrane [1–5], although the precise localization was demonstrated unequivocally only for erythrocytes [3,6]. These data show that also in FELCs (poly)phosphoinositide phosphorylation occurs in the plasma membrane, that this phosphorylation proceeds very fast and that it can be used therefore as a rapid and convenient labeling procedure for plasma membranes.

Abbreviations: FELC, Friend erythroleukaemic cells; DPI, phosphatidylinositol-4-phosphate; TPI, phosphatidylinositol-4,5-*bis*phosphate; PA, phosphatidate; PI, phosphatidylinositol

2. MATERIALS AND METHODS

FELCs (clone 745) were cultivated as in [12]. Cells were washed 3 times in buffer I (37°C; 150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 5 mM glucose and 5 mM tricine–NaOH (pH 7.5)). They were allowed to swell for 5 min on ice in buffer II (10 mM KCl, 1.5 mM $MgCl_2$ and 10 mM Tris–HCl (pH 7.5)) and then disrupted in the same medium with 10 strokes of the tight-fitting pestle of a glass Dounce homogeniser. Most cells were broken at this stage, the nuclei being still intact. Enough 2 M sucrose in buffer II was added to reach 0.25 M sucrose. The homogenate was centrifuged for 5 min at $480 \times g$ (4°C) and the supernatant saved. The pellet was resuspended in buffer III (0.25 M sucrose in 10 mM Tris–HCl (pH 7.5))

Published by Elsevier Biomedical Press

homogenized and centrifuged as above. The pellet (P_1) was kept and the pooled supernatants were centrifuged for 1 h at 88 000 × *g* (4°C). The supernatant (S_2) was saved; the 'crude membrane' pellet was resuspended in 4 ml buffer III with a loose-fitting Dounce pestle; [sucrose] was brought to 40% (w/v) by adding an appropriate amount of 60% (w/v) sucrose in 10 mM Tris–HCl (pH 7.5). Aliquots of 3 ml of this dense suspension were injected below a sucrose gradient made of 4 layers (2 ml each) of (w/v) 35%, 31%, 25% and 8.5% in 10 mM Tris–HCl (pH 7.5); the tubes were centrifuged for 2 h at 70 000 × *g* (4°C) in a Beckman SW-41 rotor. The 4 interfacial bands (A at 8.5%/25%, B at 25%/31%, C at 31%/35%, D at 35%/40%) and the pellet E were collected, diluted with 50 mM sucrose in 10 mM Tris–HCl (pH 7.5) and recentrifuged for 45 min at 88 000 × *g*. These fractions were then resuspended in 25 mM sucrose–10 mM Tris–HCl (pH 7.5) and assayed for protein [13] and enzyme activity.

Marker enzyme assays are carried out following established procedures, referred to in parentheses: 5′-nucleotidase (EC 3.1.3.5) [14], the released P_i being measured as in [15]; alkaline phosphodiesterase I (EC 3.1.4.1) [16]; alkaline phosphatase (EC 3.1.3.1) [14]; NADH-diaphorase (EC 1.6.99.3) [17]; antimycin-insensitive NADH–cytochrome *c*-reductase (EC 1.6.2.4) [14]; succinate-dehydrogenase (EC 1.3.99.1) [18]; β-*N*-acetylglucosaminidase (EC 3.2.1.30) [19]; β-D-glucuronidase (EC 3.2.1.31) [14].

(poly)Phosphoinositide phosphorylation was done as follows: Cells were washed and suspended in buffer I at 100 × 10^6 cells/ml and kept on ice; 1 ml cells was preincubated for 5 min at 37°C, then added to 200 μCi carrier-free $^{32}P_i$ in 1 ml buffer I pre-equilibrated at 37°C. Incubation was for 2 min; labeled cells were pooled with the remaining cells (~3–4 ml), diluted with cold buffer I and centrifuged for 5 min at 750 × *g*. The supernatant was thoroughly decanted and cells were then swollen and fractionated as above, except that buffers II and III contained 1 mM EGTA and that the sucrose gradient was made 0.1 mM in EGTA. Extraction of lipids and high performance thin-layer chromatography were as in [20]. After iodine staining, the silica-gel spots corresponding to TPI, DPI, phosphatidylinositol, phosphatidic acid and to the bulk phospholipids, were scraped off, suspended in 5 ml Emulsifier scintillator 299 (Packard) and counted. Only traces of radioactivity remained at the origin of the chromatogram, indicating that all the ^{32}P in the extract was bound to lipids. When (poly)phosphoinositide phosphorylation was studied in isolated membrane fractions the reaction mixture consisted of membrane fractions (10–20 μg protein) in 40 μl 50 mM Na^+-acetate, 10 mM $MgCl_2$ (pH 6.5). This suspension was preincubated for 2 min at 30°C. Phosphorylation was started by addition of 10 μl 38.8 μM ATP (disodium salt) containing 2.5 μCi [γ-^{32}P]ATP (NEN Batch 1569–256, 2900 Ci/mmol) in the acetate buffer. After vortexing for 15 s, the reaction was stopped by addition of 2 ml ice-cold acidic chloroform/methanol mixture and lipids extracted and separated as in [20].

3. RESULTS AND DISCUSSION

The distribution of typical membrane marker enzymes in the different subcellular fractions are expressed as De Duve plots (fig.1). For sake of simplicity, the distribution of only one marker enzyme for each subcellular fraction is presented; the distribution patterns of all plasma membrane marker enzymes studied were similar, as were also those of microsomal and lysosomal markers. The 3 plasma membrane markers 5′-nucleotidase, alkaline phosphodiesterase I and alkaline phosphatase were enriched 19–29-fold in fraction C, which accounted for ~0.8–1% of the total homogenate protein. Fraction B showed a similar enrichment in these markers but represented only 0.2–0.4% of the cell protein. The comparable degree of enrichment of the 3 plasma membrane markers in fraction C suggests that this fraction is representative for the whole plasma membrane. For the same reasons, fraction B also seems to consist of plasma membrane material, its lower sedimentability being attributed to a somewhat higher lipid/protein ratio than in fraction C. Fraction C showed very little contamination by material derived from the endoplasmic reticulum, as indicated by the low activities of both NADH–diaphorase and antimycin-insensitive NADH–cytochrome *c*-reductase. Mitochondrial contamination, assessed by the succinate-dehydrogenase activity, was not detectable in either of the fractions B and C. Mitochondrial breakage during cell homogenization might

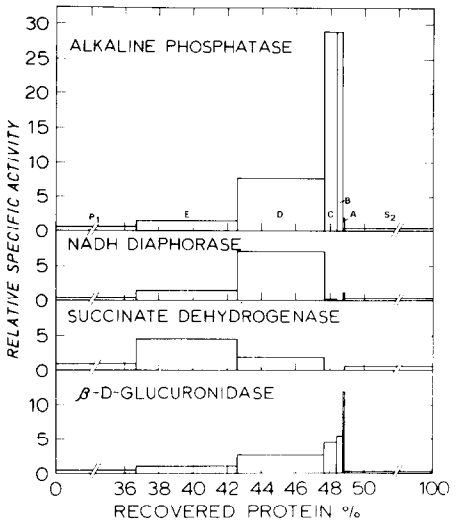

Fig.1. Distribution of various marker enzymes in the different membrane fractions of FELC homogenates. The relative specific activities are plotted against the percentage of recovered protein in each fraction which are denoted as: P_1, unbroken cells, nuclei; E, fraction enriched in mitochondrial membranes; D, microsomal membranes; B and C, plasma membrane fraction; A, lysosomal membranes; S_2, soluble proteins. Relative specific activities are expressed as recovered activity/mg recovered protein in each fraction divided by activity/mg protein in the homogenate. The relative specific activity in the homogenate = 1.0.

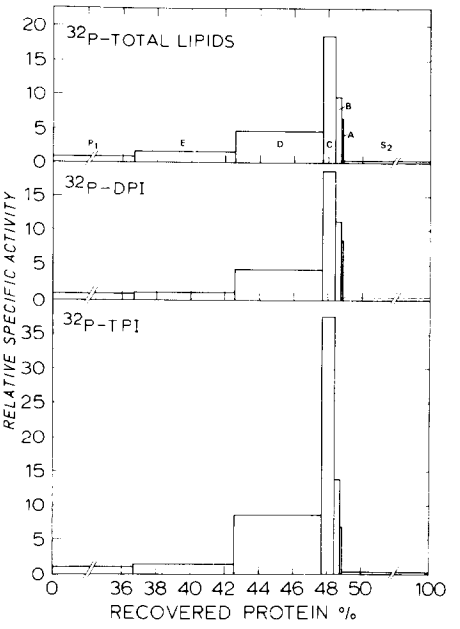

Fig.2. Distribution of the ^{32}P radioactivity in the total lipid extracts and in the newly phosphorylated DPI and TPI in the different membrane fractions of FELC homogenates. The relative specific activities are plotted against the percentage of recovered protein in each fraction. Symbols are as in fig.1.

explain the presence of succinate-dehydrogenase activity in the S_2 fraction (soluble cell components).

The presence of both β-D-glucuronidase (fig.1) and β-*N*-acetyl-glucosaminidase (not shown) in fraction C, indicated that some lysosomal membranes still contaminated the plasma membrane. Altogether, these data show that reasonably pure plasma membranes can be isolated from FELCs as a fraction which accounts for ~ 1% of the total cell protein and 15% of the total plasma membrane markers. In addition, this plasma membrane fraction was characterized by high amounts of cholesterol, sphingomyelin and phosphatidylserine when compared to the whole cell. Detailed analyses of the lipid composition of the various membrane fractions will be published elsewhere.

When FELCs were incubated in the presence of $^{32}P_i$ for 2 min they readily incorporated the label into proteins and phospholipids. Lipid analyses on the cell homogenate showed that the newly phosphorylated DPI and TPI accounted for 72% of the total ^{32}P cpm. Phosphatidic acid, phosphatidylinositol and the bulk of other phospholipids (including phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine and sphingomyelin) incorporated 19, 3 and 6% of the total radioactivity, respectively. This indicates that under the experimental conditions used, most of the label was used for (poly)phosphoinositide phosphorylation and that the contribution of the de novo phospholipids biosynthesis pathway was comparatively small. In the plasma membrane (fraction C), up to 94% of the incorporated ^{32}P was found in DPI (51%) and TPI (43%); no label was detectable in PI, whereas phosphatidic acid accounted for 2.4% and the bulk phospholipids for 2.6% only. The only significant incorporation of ^{32}P into bulk

phospholipids (26% of the total radioactivity) was found in fraction E. On the other hand, fraction E had only 34% of the label in DPI and 18% in TPI, whereas phosphatidic acid contained 19% of the label.

Fig.2 shows the distribution over the various membrane fractions of the ^{32}P present in the total lipid extracts as well as in DPI and TPI. It is obvious that the plasma membrane (fraction C) shows the highest enrichment in (poly)phosphoinositide phosphorylation (19-fold in DPI and 38-fold in TPI). Also, fraction B shows enrichment in DPI and TPI labeling, be it to a lesser extent than one would expect on the basis of marker enzyme enrichment. In the other fractions (P_1, E, D, A and S_2), the extent of labeling is small and very similar to the distribution of plasma membrane markers, suggesting that plasma membrane fragments are actually contaminating these fractions.

FELC homogenates and isolated membrane fractions B, C, D and E were also assayed for phosphatidylinositol-kinase and DPI-kinase activities using [γ-^{32}P]ATP under the experimental conditions in [20], which allow phosphorylation to proceed within seconds. In all fractions, DPI and TPI together accounted for most of the lipid-bound radioactivity (homogenate, 79%; B, 93%; C, 96%; D, 88% and E, 72%, see table 1). From the specific radioactivity of the [γ-^{32}P]ATP and the amounts of cpm present in [^{32}P]DPI and [^{32}P]TPI, the specific activities of both the phosphatidylinositol-kinase and DPI-kinase could be calculated (table 1). Fraction C showed the highest enrichment in phosphatidylinositol-kinase activity (12-fold), whereas DPI-kinase was found mostly enriched in fraction B (4-fold). The presence of kinase activities in fractions D and E indicate again that these two fractions are contaminated by plasma membrane fragments. The kinase activities of isolated plasma membrane fractions B and C showed lower enrichments as compared to those obtained when whole cells were first phosphorylated, then fractionated (cf. table 1 and fig.2). Whether this difference represents a selective loss of kinases during fractionation, a substrate depletion due to ATPase activity and/or a suboptimal labeling [γ-^{32}P]ATP in fractions B and C due to the presence of closed, ATP-impermeable plasma membrane vesicles, cannot be established yet. In addition, it should be emphasized that no attempt was made to determine the optimal conditions required for measuring these activities.

That (poly)phosphoinositide synthesis and breakdown occurs at the plasma membrane level, has been clearly shown only for the erythrocyte [3,6]. However, considerable uncertainty still remains concerning the subcellular localization of (poly)phosphoinositide metabolism in nucleated cells [6–11]. This paper shows that in FELCs the plasma membrane is the site of (poly)phosphoinositide synthesis and that both phosphatidylinositol-kinase and DPI-kinase are (at least in part) localized in that membrane. The above results, therefore, suggest that (poly)phosphoinositide phosphorylation in whole cells could be a method of choice to specifically label the plasma mem-

Table 1

Specific activities of phosphatidylinositol-kinase and DPI-kinase in various membrane fractions isolated from FELCs

Fraction	Phosphatidylinositol kinase (pmol . mg protein^{-1} . min^{-1})	% cpm in DPI	DPI-kinase (pmol . mg protein^{-1} . min^{-1})	% cpm in TPI
Homogenate	3.25	46.3	1.15	32.7
B	21.24	65.2	4.57	28.0
C	39.55	82.9	3.40	14.2
D	19.64	75.0	1.74	13.3
E	17.84	58.6	2.17	14.3

In addition it is shown which percentage of the total ^{32}P incorporated in the phospholipid is present in DPI and TPI, respectively

brane of nucleated cells grown in culture. In addition to its simplicity, this method has the advantage that labeling of the plasma membrane occurs through regular metabolic processes in the intact cell, thus preventing possible artifacts due to cell rupture.

ACKNOWLEDGEMENTS

This investigation was supported by a grant from the Koningin Wilhelmina Fonds for Cancer Research in The Netherlands, and by the Swiss National Fund for Scientific Research (grant 83.834.0.80 to A.R.). We thank Dr F. van Mansveld for a generous gift of [γ-^{32}P]ATP.

REFERENCES

[1] Michell, R.H., Harwood, J.L., Coleman, R. and Hawthorne, J.N. (1967) Biochim. Biophys. Acta 144, 649–658.
[2] Griffin, H.D. and Hawthorne, J.N. (1978) Biochem. J. 176, 541–552.
[3] Garrett, R.J.B. and Redman, C.M. (1975) Biochim. Biophys. Acta 382, 58–64.
[4] Michell, R.H. and Hawthorne, J.N. (1965) Biochem. Biophys. Res. Commun. 21, 333–338.
[5] Kai, M., White, G.L. and Hawthorne, J.N. (1966) Biochem. J. 101, 328–337.
[6] Allan, D. and Michell, R.H. (1978) in: Cyclitols and Phosphoinositides (Wells, W.W. and Eisenberg, F. jr, eds) pp. 325–336, Academic Press, New York.
[7] Kai, M., Salway, J.G. and Hawthorne, J.N. (1968) Biochem. J. 106, 791–801.
[8] Sheltawy, A., Brammer, M. and Borrill, D. (1972) Biochem. J. 128, 579–586.
[9] Keough, K.M.W. and Thompson, W. (1972) Biochim. Biophys. Acta 270, 324–336.
[10] Oron, Y., Sharoni, Y., Lefkovitz, H. and Selinger, Z. (1978) in: Cyclitols and Phosphoinositides (Wells, W.W. and Eisenberg, F. jr, eds) pp. 383–397, Academic Press, New York.
[11] Eichberg, J. and Hauser, G. (1973) Biochim. Biophys. Acta 326, 210–223.
[12] Storm, G., Bosman, G.J.C.J.M., Boer, P., Roelofsen, B., Op den Kamp, J.A.F. and Van Deenen, L.L.M. (1982) Biochem. Internat. 5, 169–176.
[13] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, M.J. (1951) J. Biol. Chem. 193, 265–275.
[14] Evans, W.H. (1979) in: Laboratory Techniques in Biochemistry and Molecular Biology (Work, T.S. and Work, E., eds) vol. 7, pp. 1–259, Elsevier Biomedical, Amsterdam, New York.
[15] Baginski, E.S. and Zak, B. (1960) Clin. Chim. Acta 5, 834–838.
[16] Aronson, N.N. and Touster, O. (1974) in: Methods in Enzymology (Fleischer, S. and Packer, L., eds) vol. 31, pt. A, pp. 90–102, Academic Press, London, New York.
[17] Kusaka, I. and Kitihara, K. (1967) Biochim. Biophys. Acta 148, 558–560.
[18] Earl, D.C.N. and Korner, A. (1965) Biochem. J. 94, 721–734.
[19] Kornfeld, R. and Siemers, C. (1974) J. Biol. Chem. 249, 1295–1301.
[20] Jolles, J., Zwiers, H., Dekker, A., Wirtz, K.W.A. and Gispen, W.H. (1981) Biochem. J. 194, 283–291.