FEBS Letters 356 (1994) 299–301

FEBS 14889

A 66-kDa protein associated with epidermal growth factor receptor is a proteolytic fragment of phosphoinositide-specific phospholipase C

N.D. Medvedeva*, V.Yu. Alexeyev, N.V. Tsupkina, N.N. Nikolsky

Institute of Cytology, Tichoretsky ave. 4, St. Petersburg 194064, Russian Federation

Received 4 November 1994

Abstract It is shown that in the A431 cells, EGFR is co-immunoprecipitated with a group of proteins recognized by antibodies to phospholipase Cγ. These are 145- and 47-kDa proteins corresponding to phospholipase Cγ and Nck, respectively, and an unidentified 66-kDa protein. The association of phosphoinositide-specific phospholipase Cγ and 66-kDa protein to EGFR was observed in the A431 cells with or without the EGF treatment. Trypsin peptide maps of these two proteins are similar so it is assumed that the 66-kDa protein is related to phospholipase Cγ.

Key words: Epidermal growth factor receptor; Phospholipase C; A 431 cell

1. Introduction

Phospholipase Cγ1 (PLCγ1) is a member of a family of PLCs which plays a central role in cell signal transduction. PLCγ1 takes part in phosphoinositide metabolism, simultaneously generating two second messengers, inositol triphosphate and diacylglycerol, triggering Ca^{2+} release from intracellular sources and activation of protein kinase C followed by numerous cellular responses (for review see [1] and references therein). PLCγ1 binds to EGFR and PDGFR via its src homology 2 (SH2) domain [2]. Association of PLCγ1 with growth factor receptors leads to its phosphorylation by the receptor, tyrosine kinase [3]. Regulation of PLCγ1 activity by tyrosine phosphorylation has been a subject of some recent articles [3,4].

The existence of another member of the PLC family, PLCα (molecular mass 62–68 kDa) has been debated as its sequence was published but the enzyme was not expressed in heterologous cells and showed no sequence homology to other PLCs, including absence of the catalytic domains [5]. Nevertheless the 62–68 kDa PLCs have been isolated from a variety of cells.

2. Materials and methods

2.1. Cell culture and treatment

A431 cells were cultured in Dulbecco's minimal essential medium (DMEM) with 10% (v/v) fetal calf serum (Gibco) at 37°C. Cells were seeded in 90 mm diameter dishes (Nunc) and grown until about 80% confluent, then shifted in to DMEM containing 0.5% fetal calf serum for 15–18 h before the addition of EGF. Mouse EGF was obtained from Sigma. A431 cells were treated with 100 ng/ml EGF in DMEM, containing 0.1% BSA and 200 mM HEPES, pH 7.4, for 5 min at 37°C.

2.2. Antibodies and immunoprecipitation

Monoclonal anti-EGFR antibody 5A9 was developed in our laboratory and has been described elsewhere [8]. Polyclonal antibody to c-terminal region of PLCγ1 (1249–1262 amino acid residues) was kindly provided by Dr. G. Carpenter [9]. Polyclonal antibody to SH2–SH3 containing the PLCγ1 domain and monoclonal anti-EGFR antibody (Mab 108) were kindly donated by Dr. J. Schlessinger [10].

*Corresponding author. Fax: (7) (812) 247 0341.

Abbreviations: EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; PDGFR, receptor of platelet-derived growth factor; PLC, phosphoinositide-specific phospholipase C; Nck, a cytoplasmic protein consisting of src homology units SH2 and SH3 [7,13].

Resting or EGF-treated cells were washed twice with ice-cold phosphate buffered saline (pH 7.4) and lysed with RIPA buffer according to Margolis et al. [11]. EGFR and PLCγ1 were immunoprecipitated with anti-PLCγ1 or anti-EGFR antibodies with the addition of 10 μl of protein A-Sepharose for 2 h at 4°C.

2.3. Immunoblotting

Immunocomplexes were boiled in Laemmli buffer, separated by SDS-PAGE and probed with anti-PLCγ1 antibodies. GAR-biotin and alkaline phosphatase detection systems (Dako) were used.

2.4. Peptide mapping of immunoprecipitates

Immunoprecipitates, obtained with anti-PLCγ1 antibodies from A431 cell lysates, were electrophoresed in 7% polyacrylamide gels and bands corresponding to PLCγ1 and 66-kDa protein were cut out, digested by modified trypsin (Sigma), subjected to 17% SDS-PAGE, and silver stained according to Cleaveland et al. [12].

3. Results and discussion

It is known that PLCγ1 can associate with growth factor receptors. PLCγ1 was previously shown to be co-immunoprecipitated with EGFR using anti-PLCγ1 antibodies. Some types of monoclonal antibodies to PLCγ1 can recognize Nck and other proteins containing an epitope common to PLCγ1 [13]. In our experiments, EGFR was immunoprecipitated from A431 cells lysates, isolated by SDS-PAGE, transferred to nitrocellulose and subjected to Western blot with anti-PLCγ1 antibodies (Fig. 1). In untreated cells, a 145-kDa band corresponding to PLCγ1 was detected along with 66- and 60 kDa proteins. There are contradictory data concerning the association of PLCγ1 with EGFR in untreated A431 cells. Margolis et al. [11] found EGFR co-immunoprecipitated with anti-PLCγ1 antibodies in untreated A431 cells, but this association was not observed in NIH 3T3 cells [14]. In our experiments, co-immunoprecipitation was seen with either monoclonal or polyclonal antibodies to EGFR and with two types of anti-PLCγ1 antibodies. It may be assumed that some of the EGFR molecules remain phosphorylated and bind PLCγ1. Treatment of A431 cells with EGF failed to induce any significant changes in the protein set associated to EGFR and recognized by anti-PLCγ1 antibodies (Fig. 1).

To determine whether 60- and 66-kDa proteins specifically bind the anti-PLCγ1 antibodies, PLCγ1 was precipitated from

SSDI 0014-5793(94)01276-8

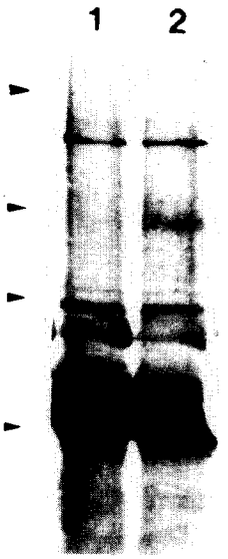

Fig. 1. Co-immunoprecipitation of PLCγ1 and EGFR. A431 cells were lysed without (1) and with (2) EGF treatment, precipitated with anti-EGFR antibody and blotted with anti-PLCγ1 antibody. Arrows indicate the molecular weight standards from top to bottom: 170 kDa, 97 kDa, 68 kDa, 45 kDa.

A431 cells with or without EGF treatment and blotted using the same antibodies (Fig. 2).

The HL-60 cells containing PLCγ2 but not PLCγ1 were used as a control. In A431 cells, 145- and 66-kDa bands were observed. None of them were detected in HL-60 cells (not shown).

It has been shown previously that unidentified 65-kDa protein is co-immunoprecipitated with anti-PDGFR in PAE cells [15]. This protein appeared to be a major component in quiescent and PDGF-treated cells [16]. A protein with the same molecular mass was detected by monoclonal antibodies to PLCγ1 in different experimental models. The 66-kDa protein was bound to B-16-5 monoclonal anti-PLCγ1 antibodies from

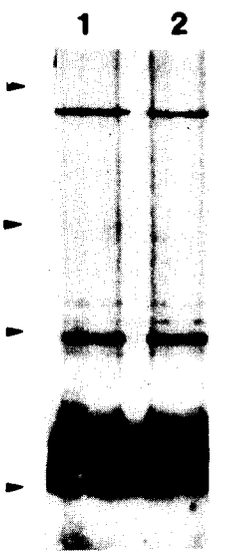

Fig. 2. Western blots of PLCγ1 from A431 cell lysates. PLCγ1 was immunoprecipitated with anti-PLCγ1 antibody from A431 cell lysates without (1) or with (2) EGF treatment and blotted with the same antibody. Arrows indicate the molecular weight standards from top to bottom: 170 kDa, 97 kDa, 68 kDa, 45 kDa.

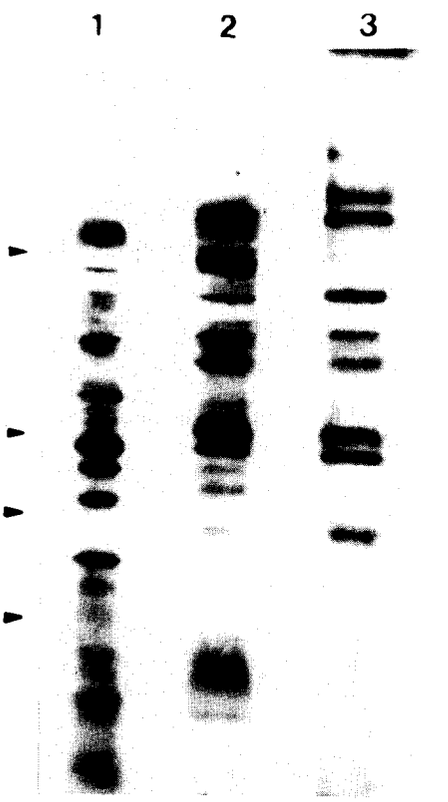

Fig. 3. Trypsin peptide mapping of the Nck (1), 66-kDa protein (2) and PLCγ1 (3). Arrows indicate the molecular weight standards from top to bottom: 45 kDa, 29 kDa, 20 kDa, 14.4 kDa.

rat brain homogenates [13]. PLCγ1 and an additional 65–70 kDa protein were found in PC-12 cell nuclei using a mix of monoclonal antibodies to PLCγ1 [16].

These proteins could be either co-immunoprecipitated with PLCγ1 or related to it. To examine the possible relationship between these two proteins, digestion with trypsin and peptide mapping were carried out. The pattern of major polypeptides of PLCγ1 and 66-kDa protein were similar and differed from Nck, a 47-kDa protein also recognized by anti-PLCγ1 antibodies (Fig. 3).

In our experiments a mixture of protease inhibitors was used and all procedures were carried out on ice so that the appearance of the 66-kDa protein could hardly be a result of in vitro degradation of PLCγ1. It seems more probable that such degradation occurs in living cells.

It is known that in A431 cells a small amount of PLCγ1 is associated with the EGFR. We have shown that its 66-kDa fragment is also associated with the receptor. One may suggest that this association plays some role in the EGFR–PLCγ1 interaction. The binding sites of the 66-kDa protein and its possible role in cell signal transduction are now under study.

Acknowledgements: This work was supported in part by the Russian Basic Research Foundation (N 9304-21708). We would like to thank Dr. M. Diakonova for valuable advice.

References

[1] Rana, R.S. and Hokin, L.E. (1990) Physiol. Rev. 70, 115–164.
[2] Majerus, P.W. (1992) Annu. Rev. Biochem. 61, 225–250.

[3] Rhee, S.G. and Choi, K.D. (1992) J. Biol. Chem. 267, 12393–12396.
[4] Wahl, M.I., Jones, G.A., Nishibe, Sh., Rhee, S.G. and Carpenter, G. (1992) J. Biol. Chem. 267, 10447–10456.
[5] Rhee, S.G., Suh, P.G., Ryu, S.H. and Lee, S.Y. (1992) Science 244, 546–550.
[6] Cockroft, S. and Thomas, G.H. (1992) Biochem. J. 288, 1–14.
[7] Lehmann, J.M., Riethmuller, G. and Johnson, J.P. (1990) Nucleic Acids Res. 18, 1048.
[8] Sorokin, A., Nesterov, A., Sorkin, A., Ignatova, T. and Galaktionov Kudryavtseva, N. (1989) Tsitologia 31, 549–555.
[9] Arteaga, C., Johnson, M., Todderud, G., Coffey, R., Carpenter, G. and Page, D. (1991) Proc. Natl. Acad. Sci. USA 88, 10435–10439.
[10] Bellot, F., Moolenaar, W., Kris, R., Mirakhur, B., Verlaan, I., Ullrich, A., Schlessinger, J. and Felder, S. (1990) J. Cell Biol. 110, 491–502.
[11] Margolis, B., Rhee, S.G., Felder, S., Mervic, M., Lyall, R., Levitzky, A., Ullrich, A. and Schlessinger, J. (1989) Cell 57, 1101 1107.
[12] Cleaveland, D.W., Fisher, S.G., Kirshner, M.W. and Laemmli, U.K. (1977) J. Biol. Chem. 252, 1102–1106.
[13] Park, D. and Rhee, S.G. (1992) Mol. Cell. Biol. 12, 5816–5823.
[14] Meisenhelder, J., Rhee, S.G. and Hunter, T. (1989) Cell 57, 1109–1122.
[15] Valius, M., Kazlauskas, A. (1993) Mol. Cell. Biol., 13, 133–143.
[16] Erriksson, A., Siegbahn, A., Westermark, B., Heldin, C.H. and Claesson-Welsh, L. (1992) EMBO J. 11, 543–550.
[17] Taylor, G.D., Fee, J.A., Silbert, D.F. and Hofmann, S.L. (1992) Biochem. Biophys. Res. Commun. 188, 1176–1182.
[18] Mazzoni, M., Bertangolo, V., Neri, L.M., Carinic, Marchisio, M., Milani, D., Manzoli, F.A. and Capitani, S. (1992) Biochem. Biophys. Res. Commun. 187, 114–120.