gp 140, the C3d/EBV receptor (CR2), is phosphorylated upon in vitro activation of human peripheral B lymphocytes

Monique Barel, Aimé Vazquez+, Christiane Charriaut, Maria Thérèse Aufredou+, Pierre Galanaud+ and Raymond Frade*

Laboratoire de Biochimie des Antigènes de Membrane, INSERM U.139, Hôpital H. Mondor, 94010 Creteil and *+Université de Paris-Sud, INSERM U.131, Clamart, France*

Received 2 December 1985; revised version received 17 January 1986

gp 140, the C3d/EBV receptor (CR2), is a specific marker of human B lymphocytes. Very recent data suggest that CR2 is a membrane site involved in early B cell activation. These properties of CR2 led us to analyze the molecular events associated with gp 140. We analyzed whether in some conditions of B lymphocyte activation, CR2 could be phosphorylated. We have found that when highly enriched peripheral B cells were cultured for 48 h with anti-μ Ab and/or SAC, in order to provide an optimal activating signal, phosphorylation of the CR2 was induced.

Complement receptor *Virus receptor* *C3d* *Epstein-Barr virus* *Phosphorylation* *B lymphocyte* *Lymphocyte activation*

1. INTRODUCTION

gp 140 was originally isolated [1] as the C3 receptor of the human B lymphoma cell line, Raji, and identified as a membrane C3b binding component [1,2], although the part of the C3b molecule involved in the gp 140-C3b interaction was not determined. Later gp 140 was described as carrying a C3d receptor activity [3]. Further molecular analysis showed that gp 140 binds to the C3d site expressed on the C3d, C3dg and C3bi fragments, and to a lesser extent on the C3b fragment [4].

*To whom correspondence should be addressed

Abbreviations: gp 140, 140 kDa membrane glycoprotein; C3, native third component of complement; C3b, 175 kDa fragment of trypsin cleaved C3; C3d, the 33 kDa fragment of C3; EBV, Epstein-Barr virus; CR2, C3 receptor type 2, B cell receptor specific for C3d, C3dg, C3bi≫ C3b; BCGF, B cell growth factor; SAC, *Staphylococcus aureus* of the Cowan I strain; anti-μ Ab, anti IgM antibodies; sIgG, surface immunoglobulins; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; NP-40, Nonidet P-40

Thus, gp 140 is the human C3d receptor (CR2) which is a specific marker of human B lymphocytes.

gp 140, the CR2, is recognized by the polyclonal antibodies prepared against the highly purified receptor [3] but also by some monoclonal antibodies such as B2 [5,6], HB-5 [7,8] and OKB-7 [9,10] prepared randomly against the whole B lymphocytes.

CR2 was also found, by us [11] and others [9, 12], to be the EBV receptor. Meanwhile, we have found that F(ab)'2 anti-gp 140 enhances, in the presence of BCGF, the proliferation of peripheral human B lymphocytes [13]. Taken together, these data lend support to CR2 being a specific membrane site involved in early B cell activation in synergy with other membrane and/or intracellular events induced by growth factors such as BCGF or by multipotent agents such as EBV. Such functions of CR2 led us to analyze the molecular events associated with gp 140. Thus, among all the putative properties of CR2 involved in B cell activation, phosphorylation of this receptor needs to be analyzed.

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/86/$3.50

Here we attempted to determine whether phosphorylation of CR2 could be induced in human B lymphocytes. We have found that gp 140, the CR2, is phosphorylated upon in vitro activation of peripheral B lymphocytes.

2. MATERIALS AND METHODS

2.1. *Cells*

Blood was drawn from healthy volunteers and peripheral blood mononuclear cells were isolated by centrifugation on Ficoll-Hypaque (Pharmacia). B enriched cells were prepared by two successive cycles of E-rosette-forming cell depletions using aminoethylisothiouronium bromide hydrobromide (AET)-treated sheep erythrocytes and one cycle of plastic adherence. The resulting preparations represented about 5% of the initial mononuclear cells and will be referred to as highly enriched B cells. They contained more than 70% membrane Ig^+ cells, 5–15% peroxidase-positive cells and less than 0.5% E-rosette-forming cells or $T3^+$ cells (using OKT3 monoclonal antibody, Ortho Pharmaceutics, Raritan, NJ). They did not proliferate when stimulated with various concentrations of phytohemagglutinin.

2.2. *Activation of B lymphocytes*

50×10^6 highly enriched B cells were cultured for 48 h in RPMI 1640 medium containing 2-mercaptoethanol and 10% fetal bovine serum in a final volume of 24 ml without stimulus or with 10 μg/ml of immobilized anti-μ Ab (BioRad) and/or SAC (1/10000 final concentration).

2.3. *Phosphorylation of human B lymphocytes*

Phosphorylation was performed as described by Pober et al. [14]. Briefly, B lymphocytes (50×10^6) were incubated for 1 h at 37°C in buffer A (150 mM NaCl, 5 mM $MgCl_2$, 5 mM KCl, 2 mM L-glutamine, 1.8 mM glucose, 10 mM Tris, pH 7.4) to deplete cells of P_i. Cells were pelleted then incubated in the presence of 5 mCi ^{32}P (185 MBq, Amersham) in 1 ml buffer A for 2 h at 37°C.

2.4. *Cell membrane extract*

Cells were extensively washed in phosphate buffered saline (PBS), pH 7.4. Then cells were lyzed by the addition of 3×10^{-4} M barbital buffer (pH 7.4) at 4°C and membranes collected by centrifugation at $100000 \times g$ for 30 min at 4°C. Membrane proteins were washed 3 times in the same buffer then solubilized with 1% NP-40 (Fluka) in barbital buffer (0.04 mM, pH 7.4) at 4°C for 45 min. Insoluble material was removed by centrifugation for 60 min at $100000 \times g$ at 4°C. The protein content of the solubilized membranes was estimated by the Bradford assay [15].

2.5. *Immunoprecipitation*

Solubilized membrane extracts were precleared by absorption on 50 mg Sepharose-protein A (Pharmacia) preloaded with 4 mg IgG of a rabbit preimmune serum for 1 h at 4°C. Effluent was absorbed for 2 h at 4°C on 15 mg Sepharose-protein A preloaded successively with rabbit IgG fraction of anti-mouse Ig (Dako, Denmark), then with 50 μl of the monoclonal OKB-7 (Ortho), an anti-CR2 [4,9,10]. Elution was performed with sample buffer (62.5 mM Tris, 1% SDS, 10% glycerol, pH 6.8) containing 2-mercaptoethanol.

2.6. *SDS-PAGE*

SDS-PAGE was performed according to Laemmli [16] on a 10% polyacrylamide gel. Gels were washed extensively by 1 M NaOH, to remove the $^{32}P_i$ non-covalently bound to membrane components. Then, gels were autoradiographed using Trimax XD films with intensifying screens.

3. RESULTS AND DISCUSSION

It is well established that the binding of anti-μ Ab with the sIgGs receptor and/or their crosslinking with agents such as SAC induce biochemical events which lead to B cell activation [17,18]. To provide an optimal activating signal for B lymphocytes, preparations of highly enriched B cells were cultured for 48 h with anti-μ Ab and SAC added together or separately. B cells cultured in the absence of these reagents were used as controls. After this step of activation, 50×10^6 cells were washed, depleted in inorganic phosphate and incubated with ^{32}P for 2 h at 37°C. Then cells were extensively washed and membranes prepared. Membrane components were solubilized in 1% NP-40 in barbital buffer, pH 7.4. Solubilized membrane components were submitted either directly to gel electrophoresis analysis or to immunoprecipitation with a monoclonal anti-CR2.

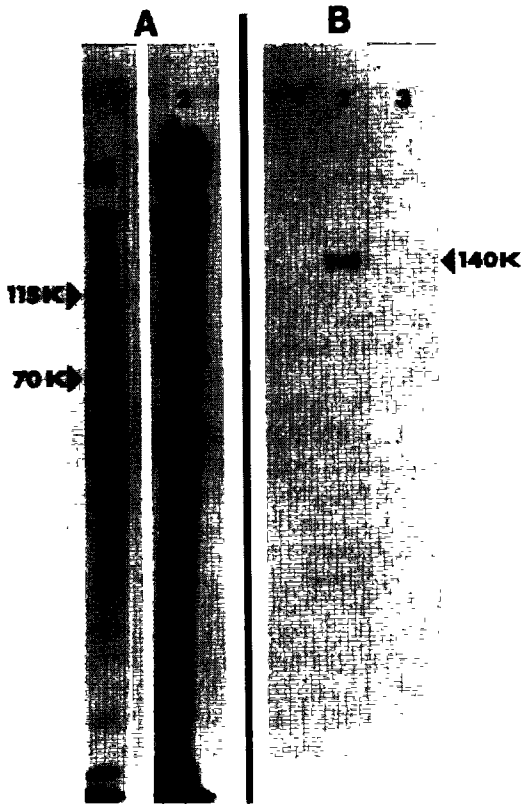

Fig.1. Analysis by SDS-PAGE of ^{32}P-labeled membrane components solubilized from human peripheral B lymphocytes. 50×10^6 cells, non-activated or activated by anti-μ Ab and/or SAC during 48 h, were labeled by 5 mCi of $^{32}P_i$ for 2 h at 37°C. Membranes were prepared then membrane components were solubilized by 1% NP-40. Panel A (lanes): 20 μl crude extract from non-activated (1) and anti-μ Ab and/or SAC activated (2) B lymphocytes were subjected to gel electrophoresis. Panel B (lanes): solubilized membrane extracts from non-activated (1) or anti-μ Ab and/or SAC activated (2) B lymphocytes were precleared by absorption on Sepharose-protein A preloaded with IgG fraction of a rabbit preimmune serum for 1 h at 4°C. Effluent was absorbed on Sepharose-protein A preloaded successively with rabbit IgG fraction of anti-mouse Ig and then with a monoclonal anti-CR2, OKB-7. Elution was performed with sample buffer containing 2-mercaptoethanol. A control was performed from activated B lymphocyte extract by measuring the eluate obtained from Sepharose-protein A loaded only with rabbit IgG fraction of anti-mouse Ig (3). Samples were run on a 10% polyacrylamide gel and analyzed by autoradiography.

As shown in fig.1, phosphorylation of solubilized membrane components occurred in non-activated and in anti-μ Ab and/or SAC activated B lymphocytes (fig.1A). However, when the same amount of solubilized membrane proteins was analyzed in the same conditions, i.e. gel electrophoresis and autoradiogram analysis during the same exposure time, membrane components solubilized from anti-μ AB and/or SAC activated B lymphocytes were found to be more intensively labeled (fig.1A). This is supported also by the ratio of trichloroacetic acid-precipitable ^{32}P radioactivity to solubilized membrane proteins which was higher in activated B lymphocytes (not shown). When CR2, the C3d/EBV receptor, was immunoprecipitated from membrane extracts of non-activated or activated B lymphocytes, using the monoclonal OKB-7, which reacts with gp 140 [4,9,10], phosphorylation of this receptor was detected only in B lymphocytes activated by anti-μ Ab and/or SAC (fig.1B, lane 2) but not in non-activated B lymphocytes (lane 1). Such phosphorylation is associated with gp 140 even after NaOH treatment of the gels, supporting a covalent binding. Thus, these data support strongly the evidence that CR2, the C3d/EBV receptor, could be phosphorylated in peripheral B lymphocytes activated in vitro by anti-μ Ab and/or SAC, two agents which crosslink slgGs [17,18].

From these data, it appears that phosphorylation of membrane components is higher in anti-μ Ab and/or SAC activated peripheral B lymphocytes than in non-activated B cells. Very likely, these effectors activate protein kinase activities. Phosphorylations induced by anti-μ Ab and/or SAC are not specific for CR2, as many other membrane proteins are labeled. Other membrane receptors have also been reported to be phosphorylated in activated human cells [19,20].

It is more likely that the P_i labeling of CR2 is mediated through an intracellular high-energy phosphorylated intermediate (e.g. ATP). We cannot rule out the possibilities that phosphorylation of CR2 is mediated either by some phosphate donor transported and utilized in the extracellular space or that an intracellular phosphorylation has taken place on an extracellular residue or a phospholipid associated with CR2. Amino acid sequence analysis of CR2 will resolve this question. However, it is more likely that an intracellular

residue of gp 140 is phosphorylated upon B cell activation. In such a hypothesis, CR2 would be a transmembrane receptor, like human histocompatibility antigens [14]. Such a transmembrane conformation would be involved in transmission of post-membrane signals, in direct or indirect CR2- enzyme and/or CR2-cytoskeletal interactions. Indeed, different proteins that bind to actin have been shown to be phosphorylated [21,22].

It appears from our and others' data that the crosslinking of gp 140, the C3d/EBV receptor (CR2), at the cell surface by probes such as F(ab)'2 anti-gp 140 [13], particle bound C3d [23] or inactivated EBV particles [24] leads to the enhancement of B lymphocyte proliferation. The level and the kinetics of CR2 phosphorylation in human B lymphocytes activated by these different probes are under analysis.

ACKNOWLEDGEMENTS

This work was supported by grants from INSERM and Association de Recherche sur le Cancer.

REFERENCES

[1] Barel, M., Charriaut, C. and Frade, R. (1981) FEBS Lett. 136, 111-114.
[2] Charriaut, C., Barel, M. and Frade, R. (1982) Eur. J. Immunol. 12, 289-294.
[3] Frade, R., Barel, M., Krikorian, L. and Charriaut, C. (1984) Eur. J. Immunol. 14, 542-548.
[4] Frade, R., Myones, B., Barel, M., Krikorian, L., Charriaut, C. and Ross, G.D. (1985) Eur. J. Immunol. 15, in press.
[5] Nadler, L., Stashenko, P., Hardy, R., Van Agthoven, A., Terhorst, C. and Schlossman, S.F., (1981) J. Immunol. 126, 1941-1947.
[6] Iida, K., Nadler, L. and Nussenzweig, V. (1983) J. Exp. Med. 158, 1021-1033.
[7] Tedder, T.F., Clement, L.T. and Cooper, M.D. (1983) Fed. Proc. Fed. Am. Soc. Exp. Biol. 42, 718 (abstr.).
[8] Weiss, J.J., Tedder, T.F. and Fearon, D.T. (1984) Proc. Natl. Acad. Sci. USA 81, 881-885.
[9] Nemerow, G.R., Wolfert, R. and Cooper, N.R. (1984) Complement 1, 163.
[10] Rao, P.E., Wright, S.D., Westberg, E.F. and Goldstein, G. (1985) Cell Imunol. 93, 549-555.
[11] Frade, R., Barel, M., Ehlin-Henriksson, B. and Klein, G. (1985) Proc. Natl. Acad. Sci. USA 82, 1490-1493.
[12] Fingeroth, J.D., Weiss, J.J., Tedder, T.F., Strominger, J.L., Biro, P.A. and Fearon, D.T. (1984) Proc. Natl. Acad. Sci. USA 81, 4510-4514.
[13] Frade, R., Crevon, M.C., Barel, M., Vazquez, A., Krikorian, L., Charriaut, C. and Galanaud, P. (1985) Eur. J. Immunol. 15, 73-76.
[14] Pober, J.S., Guild, B.C. and Strominger, J.L. (1978) Proc. Natl. Acad. Sci. USA 75, 6002-6006.
[15] Bradford, M.M. (1976) Anal. Biochem. 72, 248-253.
[16] Laemmli, U.K. (1970) Nature 227, 680-685.
[17] Muraguchi, A., Butler, J.L., Kehrl, J.H. and Fauci, A.S. (1983) J. Exp. Med. 157, 530-538.
[18] Almerigogna, F., Biagotti, R., Giudizi, G.M., Del Prete, G.F., Maggi, E., Mazetti, M., Alessi, A., Ricci, M. and Romagnani, S. (1985) Cell Immunol. 95, 358-367.
[19] Kasuga, M., Karlsson, F.A. and Kahn, C.R. (1982) Science 215, 185-186.
[20] Farrar, W.L. and Taguchi, M. (1985) Lymphokine Res. 4, 87-93.
[21] Perrie, W.T., Smillie, L.B. and Perry, S.V. (1972) Biochem. J. 128, 105P-106P.
[22] Pinder, J.C., Bray, D. and Gratzer, W.B. (1977) Nature 270, 22-29.
[23] Melchers, F., Erdei, A., Schulz, T. and Dierich, M.P. (1985) Nature 317, 264-267.
[24] Masucci, M.G., Szigetti, R., Ernberg, I. and Klein, E. (1985) Eur. Fed. Immunol. Soc., 7th Meeting, 158 (abstr.).