Phosphorylation of class I histocompatibility antigens in human B lymphocytes

Regulation by phorbol esters and insulin

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Phosphorylation of membrane proteins is one of the earliest steps in cell activation induced by growth-promoting agents. Since MHC (major histocompatibility complex) class I molecules are known to contain phosphorylation sites in their C-terminal intracellular domain, we have studied the regulation of HLA (human leucocyte antigen) phosphorylation in intact cells by two mitogens, namely TPA (12-*O*-tetradecanoylphorbol 13-acetate), a phorbol ester, and insulin, which are thought to exert their mitogenic effects through the stimulation of different protein kinases (protein kinase C and a tyrosine kinase respectively). Human B lymphoblastoid cells (526 cell line) were pulsed with [³²P]_i to label the intracellular ATP pool. Cells were then stimulated for 10 min with TPA, insulin, cyclic AMP or EGF (epidermal growth factor). The reaction was stopped by cell lysis in the presence of kinase and phosphatase inhibitors, and class I HLA antigens were immunoprecipitated with monoclonal antibodies. Analysis of labelled proteins by gel electrophoresis and autoradiography revealed that TPA increased the phosphorylation of the 45 kDa class I heavy chain by 5–7-fold, and insulin increased it by 2–3-fold. Cyclic AMP and EGF had no stimulatory effect. Analysis of immunoprecipitated HLA molecules by two-dimensional gel electrophoresis showed that TPA and insulin stimulated the incorporation of ³²P into different 45 kDa molecular species, suggesting that different sites were phosphorylated by two agents. Moreover, incubation of purified class I MHC antigens with partially purified insulin-receptor tyrosine kinase and [³²P]ATP revealed that class I antigens could also be phosphorylated *in vitro* by this tyrosine kinase. Altogether, these results therefore confirm that insulin receptors and HLA class I molecules are not only structurally [Fehlmann, Peyron, Samson, Van Obberghen, Brandenburg & Brossette (1985) Proc. Natl. Acad. Sci. U.S.A. 82, 8634–8637] but also functionally associated in the membranes of intact cells.

INTRODUCTION

Major histocompatibility complex (MHC) class I antigens consist of a family of polymorphic glycoproteins expressed on most cells of the organism. They are composed of a transmembrane 45 kDa heavy chain non-covalently associated on its extracellular domain with a smaller subunit, β_2 microglobulin. The best documented function of class I MHC antigens is to allow the recognition of foreign antigens by the T-cell receptor on cytotoxic T cells. It is now generally accepted that, on a target cell, foreign antigens and self class I molecules are co-recognized by a single receptor of the T lymphocyte. This implies that foreign antigens and MHC class I antigens interact with each other during the recognition of the target cell by the T lymphocyte. Recently, a broader role of MHC antigens has been envisioned, mainly owing to growing evidence that class I antigens are involved in hormone signalling. Indeed, monoclonal antibodies to MHC class I antigens inhibit insulin [1] and EGF [2] binding to human cultured cells and thrombin biological effects in platelets [3]. Moreover, insulin receptors and class I antigens have been shown to form selective molecular complexes in mouse liver [4,5] and cultured human lymphocytes [6].

It can thus be assumed that, besides their immunological function as restriction elements for the recognition of foreign antigens in the context of self, MHC antigens also play a role in the recognition of hormones or growth factors by their respective receptors, possibly by interacting with the latter to form 'compound' receptors [7] able to transfer the hormonal signal to the cell.

Since phosphorylation reactions play a crucial role in the transduction of hormonal or growth signals, and since class I MHC antigens contain in their cytoplasmic domain several phosphorylation sites [8], we have examined whether phosphorylation of histocompatibility class I antigens could be regulated by different effectors that are supposed to exert their mitogenic effects through the stimulation of different protein kinases.

MATERIALS AND METHODS

Cells

Two human B lymphoblastoid cell lines were used: 526 lymphocytes, kindly provided by Dr. F. M. Bach (Minneapolis, MN, U.S.A.), and IM-9 lymphocytes. Cells were maintained in RPMI 1640 medium supplemented with 10% (v/v) fetal-calf serum.

Abbreviations used: MHC, major histocompatibility complex; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; EGF, epidermal growth factor; Bt_cAMP, *N*⁶,*O*^{2'}-dibutyryl cyclic AMP; RAM, rabbit immunoglobulins against mouse immunoglobulins; PAGE, polyacrylamide-gel electrophoresis.

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Materials

Pig monocomponent insulin was generously supplied by Novo Research Institute (Copenhagen, Denmark). EGF was from Collaborative Research (Lexington, MA, U.S.A.). TPA and Bt₂cAMP were from Sigma (St. Louis, MO, U.S.A.). Aprotinin was from Specia (Paris, France). [γ -³²P]ATP (triethylammonium salt; aqueous solution, 5000 Ci/mmol) and [³²P]P_i (10 mCi/ml) were from The Radiochemical Centre (Amersham, Bucks., U.K.). Anti-HLA class I antigen mouse monoclonal antibody (W6/32), normal mouse serum and RAM were from Sebia (Issy les Moulineaux, France). Protein A from *Staphylococcus aureus* (Pansorbin) was from Calbiochem (La Jolla, CA, U.S.A.). All reagents for SDS/PAGE were purchased from Bio-Rad Laboratories (Richmond, CA, U.S.A.).

Phosphorylation of intact cells

Cells were washed and incubated in phosphate-free Krebs-Ringer bicarbonate medium [5] containing bovine serum albumin (0.5%, w/v) and [³²P]P_i (10 × 10⁶ cells, 100 μ l of medium, 100 μ Ci of ³²P) for 3 h at 37 °C. Cells were then diluted to 10 × 10⁶ cells/ml in the same medium, and effectors were added for 10 min at 37 °C. Reactions were stopped by solubilization of the cells in 50 mM-Hepes (pH 7.6)/150 mM-NaCl/0.5% Triton X-100/100 mM-NaF/20 mM-EDTA/10 mM-Na₃VO₄ containing aprotinin (1000 units/ml) for 30 min at 4 °C and centrifuged at 10000 *g* for 15 min at 4 °C. Supernatants were then used for immunoprecipitation.

Immunoprecipitation

Lysates were pre-cleared by three cycles of incubation with normal mouse serum (20 μ g/ml), RAM (100 μ g/ml) and Protein A (200 μ g/ml). Cleared lysates were then subjected to immunoprecipitation by incubation with normal mouse serum or with W6/32 (20 μ g/ml) for 3 h at 4 °C. Immune complexes were collected by incubation with Protein A for 30 min at 4 °C, followed by centrifugation at 10000 *g* for 3 min. The pellets were washed three times with 50 mM-Hepes (pH 7.6)/150 mM-NaCl/0.5% Triton X-100.

Phosphotransferase reactions

Insulin receptors were partially purified from IM-9 cells by affinity chromatography as previously described [9]. HLA class I antigens were purified by immunoprecipitation as described above. Immunoprecipitated class I antigens (3–5 μ g per assay) from 5 × 10⁶ cells were resuspended in 30 mM-Hepes (pH 7.6)/30 mM-NaCl/0.1% Triton X-100 and mixed with insulin receptors (50 ng per assay, from 10 × 10⁶ cells) and incubated without or with insulin (1 μ M) for 1 h at 15 °C before the phosphorylation assay. The phosphorylation reaction was initiated by addition of 15 μ M-[γ -³²P]ATP, 4 mM-MnCl₂ and 8 mM-MgCl₂. After 15 min at 20 °C the reaction was terminated by adding a solution containing 3% (w/v) SDS, 10% (v/v) glycerol, 10 mM-sodium phosphate, 0.01% (v/v) Bromophenol Blue and 2% (v/v) 2-mercaptoethanol. Samples were then boiled for 5 min.

Electrophoresis and autoradiography

Samples were analysed by one- and two-dimensional gel electrophoresis. One-dimensional slab-gel electro-

Fig. 1. Phosphorylation of HLA class I antigens by purified insulin-receptor protein kinase

Immunoprecipitated class I antigens were incubated with [γ -³²P]ATP without (lanes *a* and *b*) or with insulin-receptor kinase purified by affinity chromatography (lanes *c* and *d*), in the absence (lanes *a* and *c*) or in the presence of 1 μ M-insulin (lanes *b* and *d*). Reaction was stopped after 15 min at 20 °C. An autoradiogram is shown. Key: INS-R, β subunit of the insulin receptor; OR, origin.

phoresis was performed as described by Laemmli [10], with a 7.5–17.5% linear gradient of acrylamide as resolving gel. Two-dimensional gel analysis involving isoelectrofocusing (pH gradient 5–7) in the first dimension and SDS/PAGE in the second dimension (10%-acrylamide resolving gel) was performed as previously described [11]. Dried gels were exposed to Kodak X-Omat film at –80 °C.

Internalization of HLA antigens

The 526 cells, suspended at 5 × 10⁶ cells/ml in binding buffer [25 mM-Hepes (pH 7.4)/145 mM-NaCl/4 mM-KCl / 0.8 mM-MgSO₄ / 1.8 mM-CaCl₂ / 10 mM-glucose / 0.5% (w/v) bovine serum albumin] were incubated for 1 h at 37 °C without or with TPA (0.1 μ M). Cells were then washed with ice-cold binding buffer and incubated with anti-HLA class I monoclonal antibody (W6/32) or with normal mouse immunoglobulins at 20 μ g/ml for 1 h at 4 °C with gentle shaking. After two washes, cells

were incubated with ^{125}I -RAM for 1 h at 4 °C. Cells were then washed twice with binding buffer, and the radioactivity associated with the pellet was determined.

RESULTS

Phosphorylation of HLA class I antigens by purified protein kinases

The heavy chain (45 kDa) of class I HLA antigens is known to contain several cytoplasmic sites that can be phosphorylated in intact cells [12] or with purified kinases, including the Rous-sarcoma-virus tyrosine kinase (pp60^{v-src}) [13] and the cyclic AMP-dependent protein kinase [14].

In order to test whether immunoprecipitated HLA antigens could be used as kinase substrates in cell-free assays, IM-9 lymphocytes were solubilized in Triton X-100 and HLA antigens were directly precipitated with W6/32. The immunoprecipitates were then incubated with [γ - ^{32}P]ATP and insulin-receptor tyrosine kinase purified by affinity chromatography [9]. Two major phosphoproteins were detected after SDS/PAGE and autoradiography (Fig. 1, lane c): a 94 kDa component corresponding to the autophosphorylated β subunit of the insulin receptor [15] and the 45 kDa heavy chain of HLA class I antigens. The phosphorylation of both species was stimulated by insulin (Fig. 1, lane d) and the radioactivity associated with each band was not released by treating the gels with KOH (results not shown). These results thus indicate that HLA antigens from IM-9 lymphocytes can be phosphorylated *in vitro* on two different sites: a serine residue phosphorylated by the cyclic AMP-dependent protein kinase, and a tyrosine residue phosphorylated by the insulin-receptor protein kinase.

Phosphorylation of HLA class I antigens in intact cells

Since the 45 kDa heavy chain of class I antigens could be phosphorylated *in vitro* by the insulin-receptor kinase, we next investigated the possible effect of insulin on HLA phosphorylation in intact cells. Intact-cell experiments were performed with 526 B lymphocytes, a cell line expressing more HLA class I antigens than the IM-9 B-cell line [6] currently used to prepare insulin receptor [9]. The 526 lymphocytes were incubated with [^{32}P]P_i to label the intracellular ATP pool and then briefly exposed to insulin and other potential effectors. Cells were then lysed in a non-ionic detergent, and HLA antigens were immunoprecipitated and analysed by SDS/PAGE and autoradiography. Fig. 2 shows that insulin stimulated the phosphorylation of class I heavy chains by 2–3-fold (lane b), and TPA stimulated by 5–7-fold (lane e), whereas EGF (lane c) or Bt₂cAMP (lane d) had no significant stimulatory effect. 8-Bromo cyclic AMP was also tested and gave similar negative results. EGF was included as a control to test the specificity of the insulin-receptor kinase, since no detectable EGF-dependent tyrosine kinase can be detected on EBV lymphoblastoid B cells.

Class I heavy chains phosphorylated in intact cells under basal conditions were further analysed by two-dimensional gel electrophoresis. The phosphorylated 45 kDa component appeared to migrate as ten different spots, with pI values ranging from 5.2 to approx. 6.8 (Fig. 3). This heterogeneity of phosphorylated class I

Fig. 2. Regulation of HLA class I antigen phosphorylation in intact human B cells

The 526 cells were first labelled with [^{32}P]P_i for 3 h and then incubated for 10 min with the different effectors. After solubilization, HLA class I antigens were immunoprecipitated and analysed by one-dimensional SDS/PAGE and autoradiography. The effectors used were: lane a, none (control); lane b, 1 μM -insulin; lane c, 1 μM -EGF; lane d, 0.1 mM-Bt₂cAMP; lane e, 0.1 μM -TPA.

heavy chain has previously been reported by Loube *et al.* [16], who showed that the charge differences seen with ^{32}P differ from those detected after biosynthetic labelling or cell-surface iodination. It was therefore decided to investigate whether some of these heterogeneous forms could be regulated independently by the different effectors. Fig. 4 shows indeed that insulin exerts different effects on the various forms of class I heavy chains. The phosphorylation of the most acidic forms (spots 1–6 in Fig. 4) was selectively increased by insulin, whereas the phosphorylation of two forms with smaller molecular mass (spots 11 and 12) was inhibited by the hormone. The phosphorylation of class I antigens by insulin in intact cells is totally sensitive to alkali treatment.

TPA induced a different pattern, with a selective stimulation of ^{32}P incorporation into spots 11 and 12. Whether these different phosphorylated forms represent distinct glycosylated products and possibly functionally different class I antigens under the control of different protein kinases is not known, but this led us to consider the possible biological implications of HLA class I phosphorylation.

Fig. 3. Two-dimensional gel analysis of phosphorylated HLA class I antigens from 526 cells

Experimental conditions were as used for the control of Fig. 2. The immunoprecipitate was analysed by the two-dimensional gel technique, with a pH 5–7 gradient in the first dimension (isoelectrofocusing, 'IEF') and a 10 %-polyacrylamide gel in the second dimension (SDS/PAGE, 'SDS'). An autoradiogram of the gel is shown.

Fig. 4. Two-dimensional analysis of phosphorylated HLA class I antigens from 526 cells: effect of insulin and TPA

Conditions of analysis were as for Fig. 3. Cells were incubated in basal conditions, with 1 μ M-insulin or 0.1 μ M-TPA. Parts of the autoradiograms corresponding to the 45 kDa class I heavy chain are shown. The upper part of the Figure is a schematic drawing of the different spots identified on phosphorylated HLA antigens.

Effect of TPA on the internalization of HLA class I antigens

TPA induces a concomitant phosphorylation and internalization of a variety of membrane proteins, including transferrin receptors [17] and the T3-T-cell

antigen–receptor complex [18,19]. Since a large decrease of surface expression of class I antigens has been correlated with the induction of metastatic growth of certain tumours [20], the tumour-promoting activity of TPA could be mediated by a redistribution of class I antigens from the cell surface to an internal cellular pool.

Therefore, 526 lymphocytes were exposed to TPA for 1 h at 37 °C and the amount of surface class I antigens was evaluated by using mouse specific anti-class I monoclonal antibody and a labelled anti-mouse antibody. No significant difference in surface expression of class I antigens could be detected before (2800 ± 50 c.p.m. of anti-class I monoclonal antibody + ^{125}I -RAM specific binding) and after 1 h incubation with $0.1 \mu\text{M}$ -TPA (2900 ± 45 c.p.m.), a condition under which maximal phosphorylation occurs.

DISCUSSION

Among the different post-translational events that control the biological activity of proteins, phosphorylation plays a central role, mainly owing to its rapid reversibility. The constant balance existing between specific protein kinases and specific phosphatases endows the cell with a control mechanism that can be constantly switched on or off, depending on the respective activities of these enzymes. It is thus not surprising that many biological events associated with the transfer of information from the external environment to the cell involves phosphorylation reactions [21–23]. If many different kinases have been identified in the cascade of events that lead to the control of cell proliferation (cyclic AMP-dependent kinase, tyrosine kinases, protein kinase C), the nature of the proteins phosphorylated by these kinases is essentially unknown.

On the other hand, MHC class I antigens can be phosphorylated both in a cell-free assay using purified protein kinases [13,14] and in intact cells [12]. Several different phosphorylation sites have been identified on the intracellular domain of class I heavy chains, depending on the conditions used [8]. However, no physiological regulation of these phosphorylation reactions has ever been reported so far. The present study shows that, among several effectors known to activate different protein kinases, TPA and insulin were able to stimulate the phosphorylation of class I antigens in intact human B cells.

TPA, which exerts a large array of biological effects, is a potent stimulator of the phospholipid-dependent Ca^{2+} -dependent protein kinase C. TPA mimics the natural effector, diacylglycerol, by recruiting the cytosolic protein kinase to a membrane site where the enzyme phosphorylates protein substrates on serine residues [24–26]. A number of membrane proteins are phosphorylated after cell exposure to TPA [27–30]. For surface receptors, phosphorylation induced by phorbol esters is often accompanied by a desensitization phenomenon [31], sometimes owing to the internalization of surface receptors into an intracellular compartment. Concerning class I antigens, TPA, as previously reported in HL-60 cells [32], is an efficient agent in increasing their phosphorylation in human B lymphocytes. However, no translocation of surface molecules to intracellular sites could be demonstrated in these cells. This could be in agreement with a report from Loube *et al.* [16] showing that phosphorylated class I HLA molecules represent a small proportion of total cellular class I antigens. Indeed, our method used to follow the fate of cell-surface MHC antigens did not discriminate between phosphorylated and non-phosphorylated antigens.

The effect of insulin on phosphorylation of class I antigens could be more relevant to the control of cell

activation and proliferation than that induced by TPA. The β subunit of the insulin receptor contains a tyrosine kinase activity which is stimulated in the presence of insulin [15]. Autophosphorylation of the receptor is known to occur both *in vivo* and *in vitro* [33]; however, as stated above, little information is available concerning the possible endogenous substrates for this tyrosine kinase activity.

The stimulation of class I heavy-chain phosphorylation by the insulin-receptor kinase is consistent with our previous observations that these two molecules interact specifically in mouse liver membranes [4,5] and in cultured human B cells [6]. The consequences of insulin-induced class I phosphorylation are as yet difficult to determine, but should be considered in the context of the importance of tyrosine phosphorylation in the control of cell growth [34] and the appearance of insulin receptors as a marker of lymphocyte activation [35].

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REFERENCES

1. Simonsen, M. & Olsson, L. (1983) *Ann. Immunol. (Paris)* **134**, 85–92
2. Schreiber, A. B., Schlessinger, J. & Edidin, J. (1984) *J. Cell Biol.* **98**, 725–731
3. Curry, R. A., Messner, R. P. & Johnson, G. J. (1984) *Science* **224**, 509–511
4. Chvatchko, Y., Van Obberghen, E., Kiger, N. & Fehlmann, M. (1983) *FEBS Lett.* **163**, 207–211
5. Fehlmann, M., Peyron, J. F., Samson, M., Van Obberghen, E., Brandenburg, D. & Brossette, N. (1985) *Proc. Natl. Acad. Sci. U.S.A.* **82**, 8634–8637
6. Samson, M., Cousin, J. L. & Fehlmann, M. (1986) *J. Immunol.* **137**, 2293–2298
7. Simonsen, M., Skjodt, K., Crone, M., Sanderson, A., Fujita-Yamaguchi, Y., Due, C., Ronne, E., Linnet, K. & Olsson, L. (1985) *Prog. Allergy* **36**, 151–176
8. Guild, B. C. & Strominger, J. L. (1984) *J. Biol. Chem.* **259**, 9235–9240
9. Hedo, J. A., Kasuga, M., Van Obberghen, E., Roth, J. & Kahn, C. R. (1981) *Proc. Natl. Acad. Sci. U.S.A.* **78**, 4791–4795
10. Laemmli, U.K. (1970) *Nature (London)* **227**, 680–685
11. Cabral, F. & Schatz, G. (1979) *Methods Enzymol.* **56**, 602–613
12. Pober, J. S., Guild, B. C. & Strominger, J. L. (1978) *Proc. Natl. Acad. Sci. U.S.A.* **75**, 6002–6006
13. Guild, B. C., Erikson, R. L. & Strominger, J. L. (1983) *Proc. Natl. Acad. Sci. U.S.A.* **80**, 2894–2898
14. Guild, B. C. & Strominger, J. L. (1984) *J. Biol. Chem.* **259**, 13504–13510
15. Van Obberghen, E., Rossi, B., Kowalski, A., Gazzano, H. & Ponzio, G. (1983) *Proc. Natl. Acad. Sci. U.S.A.* **80**, 945–949
16. Loube, S. R., Owen, M. J. & Crumpton, M. J. (1983) *Biochem. J.* **210**, 79–87
17. May, W. S., Sahyoun, N., Jacobs, S., Wolf, M. & Cuatrecasas, P. (1985) *J. Biol. Chem.* **260**, 9419–9426
18. Cantrell, D. A., Davies, A. A. & Crumpton, M. J. (1985) *Proc. Natl. Acad. Sci. U.S.A.* **82**, 8158–8162
19. Davies, A. A., Cantrell, D. A. & Crumpton, M. J. (1985) *Biosci. Rep.* **5**, 867–876

20. Goodenow, R. S., Vogel, J. M. & Linsk, R. L. (1985) *Science* **230**, 777–783
21. Greengard, P. (1978) *Science* **199**, 146–152
22. Krebs, E. G. & Beavo, J. A. (1979) *Annu. Rev. Biochem.* **48**, 923–960
23. Cohen, P. (1982) *Nature (London)* **296**, 613–620
24. Castagna, M., Takai, Y., Kaibuchi, K., Sano, K., Kikkawa, U. & Nishizuka, Y. (1981) *J. Biol. Chem.* **257**, 7847–7851
25. Kikkawa, U., Takai, Y., Tanaka, Y., Miyake, R. & Nishizuka, Y. (1983) *J. Biol. Chem.* **258**, 11442–11445
26. Wolf, M., Le Vine, H., III, Stratford, J. R., Cuatrecasas, P. & Sahyoun, N. (1985) *Nature (London)* **317**, 546–548
27. Bourguignon, L. Y. W., Walker, G. & Bourguignon, G. J. (1985) *J. Biol. Chem.* **260**, 11775–11780
28. Hunter, T., Ling, N. & Cooper, J. A. (1984) *Nature (London)* **311**, 480–483
29. Jacobs, S., Sahyoun, N. E., Saltiel, A. R. & Cuatrecasas, P. (1983) *Proc. Natl. Acad. Sci. U.S.A.* **80**, 6211–6213
30. Takayama, S., White, M. F., Lanois, V. & Kahn, C. R. (1984) *Proc. Natl. Acad. Sci. U.S.A.* **81**, 7797–7801
31. Sibley, D. R. & Lefkowitz, J. R. (1985) *Nature (London)* **317**, 124–129
32. Feuerstein, N., Monos, D. S. & Cooper, H. L. (1985) *Biochem. Biophys. Res. Commun.* **126**, 206–213
33. White, M. F., Takayama, S. & Kahn, C. R. (1985) *J. Biol. Chem.* **260**, 9470–9478
34. Hunter, T. & Cooper, J. A. (1985) *Annu. Rev. Biochem.* **54**, 897–930
35. Helderman, J. H. (1983) *J. Immunol.* **131**, 644–648

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