Human lymphocyte activation is associated with the early and high-level expression of the endogenous lectin CSL at the cell surface

Jean-Pierre ZANETTA,*† Josiane WANTYGHEM,† Sabine KUCHLER-BOPP,* Ali BADACHE* and Michèle AUBERY†

*Laboratoire de Neurobiologie Moléculaire des Interactions Cellulaires, CNRS UPR 416, Centre de Neurochimie du CNRS, 5, rue Blaise Pascal, 67000 Strasbourg, France, and †Glycobiologie et Reconnaissance Cellulaire, INSERM U180-CNRS UAC 71, 45, rue des Saint-Pères 75270 Paris, Cedex 06, France

Lymphocytes undergo activation in response to antigens, cytokines, lectins and antibodies interacting with specific cell-surface molecules or through substances influencing signal transduction pathways. This study shows that human T- and B-cells stimulated using phorbol esters or plant lectins express early (2 h using phorbol esters and 24 h using plant lectins) a high level of a polyvalent carbohydrate-binding protein, the cerebellar soluble lectin (CSL), which is in part externalized. The lectin, immuno-

logically related to CDw70, interacts with specific glycoprotein ligands of the lymphocyte surface, including CD3 on T-cells and CD24 on B-cells. Major changes in phosphorylations associated with activation appear as largely CSL-dependent since they are specifically inhibited by anti-CSL Fab fragments. It is suggested that the lectin induces the clustering of specific cell-surface glycoproteins and plays the role of an endogenous amplifier of activation signals.

INTRODUCTION

There is a large body of evidence supporting the hypothesis that clustering of specific surface proteins is important for transducing proliferative signals [1–3]. The concept that cell-surface proteins are associated into complexes emerged from observations of their co-immunoprecipitation [4,5], but isolated complexes are not able to generate the signals obtained by cross-linking these complexes. Specific phosphorylation mechanisms occur subsequently [1–3]. Initial clustering of cell-surface molecules is not the only way to activate lymphocytes, since activation can be achieved using exogenous monovalent molecules acting on signal transduction pathways, like phorbol 12-*O*-tetradecanoyl-13-acetate (PMA) [6], or binding of apparently monovalent molecules, like interleukins [7], to specific receptors. Although the kinetics of activation differ, the activation process is accompanied by increased cell adhesiveness, implying a relationship between activation and adhesion.

The activation of T- or B-cells induced by polyvalent lectins or anti-carbohydrate antibodies suggests that clustering of surface glycoproteins is involved in polyclonal lymphocyte activation [8], but does not demonstrate a specific role of carbohydrates, since similar effects can be obtained with antibodies directed against polypeptides. However, studies using N-glycosylation inhibitors showed that T-cell activation by the plant lectin concanavalin A (ConA) [9] was enhanced by hybrid-type N-glycans produced using swainsonine but not by glucosyl-oligomannosidic N-glycans synthesized using castanospermine. Since both N-glycans bound to ConA, the differential effects validated the view that the binding of the polyvalent ConA, and the resulting cross-linking of glycoproteins, was not sufficient to induce T-cell activation. In contrast, these experiments suggested that the initial signal triggered by ConA was relayed by endogenous polyvalent carbohydrate-binding proteins capable of recognizing hybrid-type N-glycans and bridging T-cell receptor (TCR) molecules [9].

An endogenous polyvalent lectin with such a carbohydrate specificity was previously isolated from the rat cerebellum [10],

i.e. the cerebellar soluble lectin, CSL. CSL constitutes a family of high-molecular-mass homopolymers with subunits of 31.5, 33, 43, 55 and 75 kDa, representative of a cell-type specificity [11]. CSL stimulates the proliferation of normal cells [12] and is involved in cell-adhesion mechanisms [13–15]. In order to analyse the potential role of CSL in the process of lymphocyte activation, we have compared the expression and the function of CSL using two different classical ways to activate human lymphocytes: phorbol esters and plant lectins. This paper reports that CSL, immunologically related to the ‘activation-inducer molecule’ CDw70, is expressed early and at a high level during lymphocyte activation. It is suggested that CSL plays an important role in the clustering of a few cell-surface glycoproteins (including CD3 and CD24) and in the subsequent changes in the phosphorylation–dephosphorylation of specific molecules.

MATERIALS AND METHODS

Human lymphocyte isolation and cultures

Peripheral blood lymphocytes from healthy individuals or Sézary’s syndrome patients were isolated by Ficoll-Paque (Pharmacia) density-gradient centrifugation. Cells were suspended at a density of 1×10^6 cells/ml in RPMI 1640 (Sigma) containing 10% (v/v) fetal-calf serum (Gibco) in 25 cm² vented tissue-culture flasks (Falcon) and cultured in a culture oven with humidified air containing 5% CO₂. After 30 min, non-adherent cells were recovered and cultured for various periods of time. T- and B-cells from healthy individuals were separated using the rosette technique and anti-CD19 immunoadsorption respectively. CD4⁺ T-cells from patients with Sézary’s syndrome were isolated by treatment [16] with complement and the B9.2 anti-CD8 monoclonal antibody (Immunotech). For stimulation, cells were incubated for different periods of time (15 min, 30 min, 1 h, 2 h, 4 h, 8 h, 24 h, 48 h and 72 h) in the presence of activators: PMA (50 ng/ml), phytohaemagglutinin (PHA; 1 µg/ml) and *Robinia pseudoacacia* agglutinin (RPA; 1 µg/ml) [17] or CSL (1 or 3 µg/ml).

Abbreviations used: AKP, alkaline phosphatase; ConA, concanavalin A; CSL, cerebellar soluble lectin; GPI, glycosyl-phosphatidylinositol; HRP, horseradish peroxidase; NGS, normal goat serum; PHA, phytohaemagglutinin; PMA, phorbol 12-*O*-tetradecanoyl-13-acetate; RNase, ribonuclease; RPA, *Robinia pseudoacacia* agglutinin; TCR, T-cell receptor.

† To whom correspondence should be addressed.

Characterization of the CSL antigen and of its antibodies

CSL was isolated from 14-day-old rat cerebellum using a procedure involving sequential extractions of the cerebellar tissue in hypotonic buffer, followed by extraction in high-salt buffer (0.4 M NaCl) as previously described [10]. The lectin which remained bound to its insoluble endogenous ligands was solubilized in high-salt buffer containing 0.5 M mannose. For isolation of CSL from human lymphocytes, cells were stimulated for 1 h with PMA, washed in PBS and CSL was extracted using 10 mM Tris/HCl buffer, pH 7.2, containing 0.4 M NaCl and 0.5 M mannose. In each case, the solubilized material was subsequently adsorbed on columns of immobilized anti-CSL antibodies equilibrated in the same buffer and eluted at acidic pH [10]. The purified lectin was dialysed against large volumes of PBS (25 mM sodium phosphate buffer containing 0.15 M NaCl). The lectin activity was verified using agglutination-inhibition of agglutination experiments (see below) and binding to its blotted ligands (see below). The specificity of the anti-CSL antibodies (monoclonal D2.11 and polyclonal) was verified by the specific detection of CSL on whole homogenate of different tissues (including cerebellum) and by their ability to inhibit the agglutination induced by CSL (see below). Fab fragments were prepared from the IgG of the polyclonal anti-CSL antibody by digestion on immobilized papain (Sigma) followed by repetitive adsorption of Fc fragments and undigested IgG on Protein A-agarose (Sigma). Their activity was tested by immunoblotting and by their ability to inhibit CSL-induced agglutination. Control Fab fragments were prepared in a similar way from a polyclonal antibody raised in rabbits against another cerebellar lectin unrelated to CSL, termed lectin R1 [18].

Electrophoretic techniques

For blotting techniques, cells were diluted in 25 mM sodium phosphate buffer, pH 7.2, containing 150 mM NaCl (PBS) and centrifuged (400 *g* for 10 min). These operations were repeated three times and the washed cells were boiled in the Laemmli dissociating buffer, submitted to SDS/PAGE [19] in 13% polyacrylamide or polyacrylamide gradient gels (ExcelGel SDS, Pharmacia) and proteins transferred on to nitrocellulose filters [20]. Blots were visualized using the polyclonal anti-CSL antibody (diluted 1/250) or the ascite fluid of a mouse monoclonal antibody (clone D2.11; diluted 1/1000), mouse monoclonal anti-CD69 and anti-CDw70 antibodies (Immunotech; 1 μ g/ml), anti-CD3 (clone S4.1; Caltag; 1 μ g/ml) and anti-CD24 (clone ALB9; Immunotech; 1 μ g/ml) or murine monoclonal anti-phosphotyrosine antibody (4G10, UBI; 1 μ g/ml), followed by incubations with alkaline phosphatase (AKP)-labelled second antibodies (Sigma) and revealed using Nitro Blue Tetrazolium (Sigma) and 5-bromo-4-chloro-3-indolyl phosphate (Sigma) [21]. All incubations were performed in PBS containing 3% BSA and normal goat serum (NGS; Biosys; 1/20 dilution), except for anti-phosphotyrosine where PBS was replaced by 25 mM Tris/HCl buffer, pH 7.2, containing 150 mM NaCl. When quantitative data were necessary, blots were scanned with a Hoffner densitometer.

For immunoprecipitations, cells were lysed using a mixture of 1% Nonidet P-40 and 1% deoxycholate containing 1 mM PMSF and incubated overnight at 4 °C in the presence of Protein A-agarose-bound mouse anti-CD3 (clone S4.1; Caltag). The supernatant was incubated in the same conditions in the presence of Protein A-agarose-bound mouse anti-CD24 (clone ALB9; Immunotech) monoclonal antibody. The washed beads were boiled in the Laemmli dissociating buffer and the solubilized

material was subjected to SDS/PAGE and blotted on to nitrocellulose for visualization of CSL ligands.

For the detection of ligands of ConA, RPA and CSL, blots were revealed using the ConA-horseradish peroxidase (HRP) technique, the biotinylated RPA-avidin-AKP technique or the biotinylated CSL-avidin-AKP technique [22]. Saturation of the blots and incubations were performed in PBS containing 3% pBSA (BSA which has been previously treated with periodic acid [23] in order to eliminate oligosaccharides of commercial BSA preparations which could interfere with lectin staining). For ConA-HRP staining, controls of specificity were performed using inhibition of the ConA binding with mannose (0.3 M). Controls for biotinylated RPA were performed by incubating blots with boiled (30 min at 100 °C) biotinylated lectin. Controls for the specificity of CSL staining were performed using boiled (30 min at 100 °C) biotinylated CSL. In some experiments, additional controls were performed using, during the incubation and the first washing, either 50 μ g/ml ribonuclease (RNase) B (from bovine pancreas, Sigma; this glycoprotein contains Man₆GlcNAc₂ N-glycans, which are high-affinity ligands of CSL) or 5 mg/ml heparin (Sigma; heparin is also a ligand of CSL).

Immunocytochemistry

For the immunoperoxidase detection of CSL, cells (resting cells and cells stimulated by PMA, PHA and RPA for 4, 24, 48 and 72 h or cells from Sézary's syndrome patients) were fixed for 24 h at 4 °C in PBS containing 4% paraformaldehyde. Endogenous peroxidase activity was blocked by incubation for 30 min at room temperature in PBS containing 1% H₂O₂. Cells were washed repetitively in PBS (or in PBS containing 0.1% Triton X-100, for permeabilization) before immunoperoxidase labelling. Cells were incubated for 30 min in a 1/20 dilution of NGS, followed by the addition of the polyclonal rabbit anti-CSL antibodies (diluted 1/250) or the preimmune rabbit serum as a control. After 2 h, cells were washed ten times with a 3% solution of BSA in PBS by centrifugation. After incubation in a 1/20 dilution of NGS, HRP-labelled goat anti-(rabbit IgG) antibodies (Biosys; 4 μ g/ml) were added and cells incubated overnight at 4 °C. After repetitive washings in PBS, bound IgGs were revealed using the diaminobenzidine method [24].

Effects of CSL and anti-CSL Fab fragments on protein phosphorylations

For studying the effect of CSL on tyrosine phosphorylations, CSL isolated by immunoaffinity chromatography from young rat cerebella was dialysed against RPMI 1640 and used at a concentration of 1 μ g/ml. After various times in culture, unstimulated cells and cells stimulated with PMA, PHA or RPA were washed in cold PBS and boiled in the Laemmli dissociating buffer [19] before electrophoresis and immunoblotting detection with anti-phosphotyrosine antibody. For studying the effects of anti-CSL antibodies on tyrosine phosphorylations, Fab fragments (dialysed against RPMI 1640) were added at a concentration of 25 μ g/ml in the culture medium at the same time as the activator, cells were then cultured for the indicated periods of time and treated as above. In order to study total phosphorylations, cells were washed three times in phosphate-free RPMI 1640 culture medium (Sigma) containing 10% (v/v) fetal-calf serum and cultured in phosphate-free medium for 30 min. Fab fragments were dialysed against this phosphate-free culture medium and added (25 μ g/ml final concentration) to the cultures at the same time as the activator (PMA) and cells were

cultured for 1 h or 24 h. [32 P]P_i (370 MBq/ml) was added and cells were cultured for another 30 min. The cell material was precipitated in 10% trichloroacetic acid, dissolved by boiling in the Laemmli dissociating buffer and subjected to SDS/PAGE followed by transfer on to nitrocellulose, autoradiography and scanning of autoradiographs.

Inhibition of the CSL-induced agglutination of human lymphocytes by anti-CSL antibodies and CSL ligands

Blood of control individuals was collected on EDTA and lymphocytes isolated by Ficoll-Paque sedimentation. They were fixed with a mixture of 4% paraformaldehyde and 0.1% glutaraldehyde in PBS and rotated overnight at room temperature. Aldehyde groups were blocked for 1 h at room temperature in PBS containing 0.1 M ethanolamine, pH 9.0. After washing in PBS, cells were suspended in PBS containing 1% periodate-treated BSA and 5 mM EDTA. Fixed cell suspension (75 μ l) was added to 100 μ l of immunopurified rat CSL dilutions in PBS containing 5 mM EDTA in round-bottomed microwells and agglutination was observed after 4 h. For inhibition of the agglutination induced by CSL (1 μ g/ml, i.e. six times the minimal value), antibodies (anti-CSL Fab fragments and anti-CSL monoclonal D2.11) or CSL ligands were added at different concentrations and agglutination was observed under the same conditions.

RESULTS

Kinetics of expression of CSL upon lymphocyte stimulation

Immunoblotting experiments using the polyclonal or the D2.11 monoclonal anti-CSL antibodies showed that CSL was weakly expressed in quiescent human lymphocytes, as a single 31.5 kDa

Figure 1 (a) Changes in the level of CSL upon human lymphocyte stimulation and (b) electrophoretic profile (silver staining) of the immunopurified CSL isolated from young rat cerebella (lane 1) and from stimulated human lymphocytes (lane 2)

(a) Immunoblotting detection of CSL in resting cells (lane 1) or lymphocytes stimulated for 24 h with PMA (50 ng/ml), PHA (1 μ g/ml) and RPA (1 μ g/ml [18]) (lanes 2–4, respectively). Lane 5: immunoblotting control for PMA-stimulated lymphocytes. (b) Numbers on the right-hand side indicate the molecular mass. Rat CSL preparations were used for phosphorylation and agglutination studies (rat cerebellum and human lymphocyte CSL showed the same specific agglutinating activities and the same pattern of inhibition by oligosaccharides).

Table 1 Increase in CSL levels (relative to unstimulated cells) in human lymphocytes stimulated for 24 h and in lymphocytes of patients with Sézary's syndrome

Data were obtained by scanning of immunoblots: unfractionated lymphocytes (Sez total) or CD4⁺ T-cells (Sez CD4⁺) from patients with Sézary's syndrome and combined effects of PMA with PHA and RPA were also analysed. Results are expressed as increase (per mg of protein) relative to control human lymphocytes ($\pm$ S.D. obtained for three scanings of at least two different blots).

Cells	Fold increase $\pm$ S. D.
PMA (50 ng/ml)	10.0 $\pm$ 0.86
PHA (1 μ g/ml)	12.4 $\pm$ 0.65
RPA (1 μ g/ml)	12.2 $\pm$ 0.73
PMA + PHA	4.3 $\pm$ 0.36
PMA + RPA	4.5 $\pm$ 0.36
Sez total	9.8 $\pm$ 0.58
Sez CD4 ⁺	9.9 $\pm$ 0.71
Control	1.0

Table 2 Kinetics of expression of the level of CSL relative to control cells in PMA- and RPA-stimulated human lymphocytes

The data (expressed relative to the control cells at the beginning of the experiments) were obtained by scanning immunoblots. S.D. values were less than 10% except for samples stimulated for 48 h by PMA (S.D. = 19%). No significant variations were observed in non-stimulated cells. Note that the maximal level was reached after a 2 h stimulation with PMA and after a 24 h stimulation using RPA (the same kinetics were obtained with the other plant lectin, PHA; not shown). Results are expressed as the increase (per mg of protein) relative to control human lymphocytes obtained for three scanings of at least two different blots.

Time	PMA (50 ng/ml)	RPA (1 μ g/ml)
15 min	1.5	1
1 h	8.0	1.5
2 h	12.2	2.5
4 h	12.4	5.5
24 h	11.5	11.0
48 h	10.1	11.4
72 h	12.4	12.2

antigen (Figure 1). Upon stimulation for 24 h by PMA, PHA or RPA, the level of CSL increased about 10-fold (Table 1) and this level persisted up to 72 h after the initiation of stimulation. When analysing after shorter periods of time after stimulation, different kinetics of CSL expression were found to be dependent on the activator. The CSL levels increased 8-fold after stimulation for 1 h with PMA (Table 2) and the maximum level (10-fold) was reached after 2 h. Using plant lectins (RPA and PHA), the CSL accumulated more slowly since the maximal level was reached after 24 h. However, despite these differences CSL appeared as one of the earliest expressed antigens during human lymphocyte activation. It appeared much earlier than lymphocyte proliferation, which becomes important 48 h and 72 h after stimulation with PMA and PHA respectively (results not shown). In order to see if such an increased expression of CSL was specific for artificially stimulated (PMA or plant lectins) lymphocytes or could actually take place *in vivo*, we analysed the CSL levels of lymphocytes from patients with Sézary's syndrome, whose lymphocytes are constantly activated. As shown in Table 1, the high level of CSL was present both in total lymphocyte and in CD4⁺ T-cell populations of these patients, suggesting that a high CSL level is naturally occurring with lymphocyte activation.

Figure 2 Immunoperoxidase detection of CSL in resting lymphocytes (a and b) or lymphocytes stimulated for 24 h by PMA (c, e and f) or RPA (d)

(c) Control using preimmune serum instead of anti-CSL polyclonal antibody. (b–d,f) Cells were permeabilized with Triton X-100, in contrast with (a) and (e). Bar represents 3 μ m.

Immunocytochemical localization of CSL in human lymphocytes

As documented for several cell types [15], CSL is an intracellular lectin which can be externalized. Using immunocytochemical labelling of lymphocytes with polyclonal anti-CSL antibodies, it appeared that CSL was expressed at a low level intracellularly in resting cells (Figures 2a and 2b) but not at their surface. Besides the low cytoplasmic staining, a concentration was found in small intracellular dots. Upon stimulation (24 h with plant lectins and PMA), CSL accumulated intracellularly showing a strong cytoplasmic staining when cells were permeabilized with Triton X-100 (Figures 2d and 2f). When cells were not permeabilized (Figure 2e) or were observed with the electron microscope (results not shown), specific immunostaining for CSL was also found at the cell surface. This indicated that the increase in CSL expression occurred mostly intracellularly but that the lectin was in part externalized upon stimulation.

CSL glycoprotein ligands of human lymphocytes

Since CSL is a soluble protein (soluble in the absence of detergent), the surface labelling suggested that the externalized CSL was bound to its surface glycoprotein ligands as in other cell types [13,14]. The ligands were detected by incubating blots of lymphocyte lysates with biotinylated CSL and subsequent staining with AKP-labelled extravidin. As shown in Figure 3(a), CSL bound to a few glycoprotein subunits in resting cells (lane 1). The

profile of the CSL ligands was less complex than that of the ligands of the plant lectins ConA and RPA (Figure 3a, lanes 2 and 3 respectively), but minor ConA- and RPA-binding glycoproteins were detected at the same molecular masses as the major CSL ligands, below 20 kDa and below 30 kDa. None of the ConA and RPA ligands was significantly expressed at higher levels after lymphocyte stimulation (between 1 h and 72 h; results not shown). Similarly, the ligands of CSL did not vary significantly upon stimulations during the same period of time (Figure 3a, lanes 4 and 5). Two major doublet bands were detected, below 20 kDa and 30 kDa, and a minor doublet band at 70–74 kDa. Separation of T- and B-cell populations by the E-rosette technique or anti-CD19 immunoaffinity chromatography suggested that the doublet below 20 kDa was common to T- and B-cells whereas the doublet below 30 kDa was distributed between T- and B-cells (Figure 3a, lanes 6 and 7 respectively). The 74 kDa component was enriched in T-cells, the 70 kDa components in B-cells. This staining appeared to be specific since it was not obtained in control experiments.

Immunoprecipitation experiments followed by SDS/PAGE under reducing conditions, blotting and detection of CSL ligands indicated that the region below 30 kDa actually constituted a triplet. Two glycosylated forms of CD3 [25] and CD24 [26] were CSL ligands in T- and B-cells respectively (Figure 3b). These CSL ligands were also revealed on blots by anti-CD3 and anti-CD24 respectively (results not shown). It is noteworthy that CD24 is a glycosyl-phosphatidylinositol (GPI)-anchored gly-

Figure 3 Detection of the ligands of CSL, ConA and RPA, in human lymphocytes

(a) The profile of major CSL glycoprotein ligands in homogenates of control human lymphocytes (lane 1) was not significantly different from that of lymphocytes stimulated for 2 h and 72 h with PMA (lanes 4 and 5 respectively). This profile was compared with that of ConA and RPA ligands (lanes 2 and 3 respectively). CSL ligands distribute between T- and B-cells (lanes 6 and 7 respectively). Lane 8: control of the specificity of the binding of CSL to the sample shown in lane 1 (incubations were performed using boiled biotinylated CSL instead of active biotinylated CSL). Numbers on the left-hand side indicate the molecular mass values. (b) CSL ligands immunoprecipitated with anti-CD3 (lane 1) and anti-CD24 antibodies (lane 2) were revealed using biotinylated CSL (the area was enlarged four times in order to show the differences of the molecular masses).

coprotein presenting extensive sequence similarity with the P31 neuronal glycoprotein [27], identified as a major neuronal ligand of CSL involved in cell-adhesion processes in the developing nervous tissue [13,28]. The nature of the other components, particularly the low-molecular-mass ligands, remains to be determined [CSL ligands of similar molecular mass are the unique ligands of CSL found in human neutrophils (J.-P. Zanetta, M. Ikekita, C. Tranchant and J.-M. Warter, unpublished work)].

Role of the interaction of CSL with its glycoprotein ligands in lymphocyte phosphorylations

Since the identified CSL ligands were surface glycoproteins considered to be important for the activation of T-cells (CD3) and B-cells (CD24) [25,26], the cell-surface expression of the endogenous lectin CSL could be an important event in lymphocyte activation as CSL was potentially capable of clustering its surface ligands. Such specific clustering of surface molecules was shown to induce specific phosphorylations [29–31].

Figure 4 Immunoblotting studies of tyrosine phosphorylations during human lymphocyte stimulation

Detection of tyrosine-phosphorylated proteins in resting lymphocytes (24 h in culture) (lane 1), lymphocytes stimulated for 2 h with PMA (lane 2), lymphocytes stimulated for 24 h with PMA (lane 3) and 72 h with RPA (lane 4). Note the shift from a 56 kDa (± 2.7 kDa; 8 determinations) band characteristic of quiescent lymphocytes to a 59 kDa (± 3.8 kDa; 8 determinations) band characteristic of the activated cells.

Table 3 Changes in the percentage of the 56 kDa tyrosine-phosphorylated band in cultures of non-stimulated human lymphocytes or cells stimulated for different periods of time with PMA, RPA or CSL and effects of anti-CSL Fab fragments

Results obtained by scanning immunoblots stained with anti-phosphotyrosine antibody are expressed as percentages for the 56 kDa band of the intensity of the immunostaining in the sum of the 56 and 59 kDa bands. (–), anti-CSL Fab fragments were not added or were replaced by anti-R1 Fab fragments as controls. (+), anti-CSL Fab fragments were added to the culture at the beginning of the experiment. Note that anti-CSL Fab fragments increased the proportion of the 56 kDa tyrosine-phosphorylated protein characteristic of resting lymphocytes in cells stimulated by PMA and RPA, but had no effects on resting cells. Note also that the 56 kDa tyrosine-phosphorylated band disappeared after 20 min using exogenous CSL.

Stimulation	Time	56 kDa band (%)	
		(–)	(+)
None	0 h	89.7	89.7
None	2 h	89.7	89.7
None	24 h	89.7	89.7
PMA (50 ng/ml)	0 h	89.7	89.7
PMA (50 ng/ml)	2 h	10.3	80.2
PMA (50 ng/ml)	24 h	10.3	85.3
RPA (1 μ g/ml)	0 h	89.7	91.7
RPA (1 μ g/ml)	2 h	84.5	86.1
RPA (1 μ g/ml)	24 h	14.9	82.0
CSL (1 μ g/ml)	0 h	97.9	
CSL (1 μ g/ml)	10 min	44.3	
CSL (1 μ g/ml)	20 min	13.9	
CSL (1 μ g/ml)	30 min	2.1	

The tyrosine-phosphorylation pattern (revealed using an anti-phosphotyrosine antibody) was changed upon lymphocyte stimulation (Figure 4). In quiescent lymphocytes, one major tyrosine-phosphorylated protein (65 % of the total immunostaining) was detected at 56 kDa; it remained unchanged for resting lymphocytes after 72 h in culture. However, after PMA stimulation, a second tyrosine-phosphorylated band appeared at 59 kDa (Figure 4) and reached its maximal level after 2 h (Table 3), while the 56 kDa component disappeared concomitantly. Upon stimulation with the plant lectins (PHA and RPA), the 59 kDa band appeared slowly (with a maximum and persistent level at 24 h), while the 56 kDa tyrosine-phosphorylated band disappeared concomitantly (Table 3). Minor tyrosine-phosphorylated constituents, including doublets around 40 kDa and 120 kDa, appeared after 24 h of stimulation in the presence of PMA and after 72 h in the presence of RPA. Considering anti-phosphotyrosine-labelled bands, it appeared that lymphocyte stimulation was associated with the progressive change from one major tyrosine-phosphorylated band to another; the time course of this change was similar to that of CSL expression. These major tyrosine-phosphorylated bands show similar behaviour upon activation with PMA and have been described as p56^{lck} and p59^{vln} [32,33].

Assuming that CSL, by clustering surface ligands, was involved in phosphorylation mechanisms, it was expected that anti-CSL Fab fragments would abolish the changes in these tyrosine-phosphorylated bands. The addition of anti-CSL Fab fragments (or control Fab fragments) neither significantly reduced the total level of tyrosine phosphorylations both in control or stimulated cells, nor the anti-phosphotyrosine labelling of the 56 kDa band and of minor bands in resting cells. However, in lymphocytes stimulated by PMA for 2 h or by RPA for 24 h, the simultaneous addition of anti-CSL Fab fragments induced a 75 % decrease of the 59 kDa tyrosine-phosphorylated band (Table 3) with a

Figure 5 Autoradiographs of the incorporation of [32 P]P, in proteins from control lymphocytes cultured for 1 h and 24 h (lanes 1 and 3 respectively), lymphocytes stimulated using PMA for 1 h and 24 h (lanes 4 and 5 respectively) and lymphocytes stimulated for 1 h with PMA in the presence of anti-CSL Fab fragments (lane 6)

Lane 2 corresponds to a blank in which cell material was acid-precipitated at the time of addition of PMA. Scanning of autoradiographs indicated that the addition of anti-CSL Fab fragments (lane 6) reduced by 74.6% the increased phosphorylation induced by 1 h stimulation with PMA (lane 4).

simultaneous increase of the 56 kDa band (while control Fab fragments were inactive). Therefore, it was suggested that major changes in tyrosine phosphorylation accompanying lymphocyte activation were CSL-dependent. In contrast, phosphorylation mechanisms characteristic of the quiescent stage were not CSL-dependent. The direct evidence supporting such a mechanism was obtained by treating lymphocytes with exogenously added CSL (Figure 1b): the 59 kDa tyrosine-phosphorylated band replaced the 56 kDa band as early as 20 min after the addition of exogenous CSL (Table 3). This switch-over demonstrated that CSL was a molecule essential for the tyrosine phosphorylation of the 59 kDa band and the dephosphorylation of the 56 kDa tyrosine-phosphorylated band.

Since residues other than tyrosine are phosphorylated during activation, we examined the effect of anti-CSL Fab fragments on phosphate incorporation into proteins of lymphocytes stimulated with PMA. As shown in Figure 5, stimulation for 1 h with PMA increased the phosphorylations by 900%, as compared with resting cells. This increase was almost completely abolished when PMA stimulation was performed in the presence of anti-CSL Fab fragments. The decrease affected all phosphorylated proteins, but bands around 30 and 37 kDa were more affected than the others.

Agglutinating properties of CSL for human lymphocytes

When cells were stimulated with PMA, aggregation occurred rapidly (30 min) in cultures and was important at the period of time when CSL reached its maximal expression (2 h). Since CSL has been shown to be involved in cell adhesion mechanisms by forming bridges between the N-glycans of its surface glycoprotein

Table 4 Inhibition of the CSL-induced agglutination of fixed lymphocytes from normal individuals by antibodies and CSL ligands

Inhibitor	Concentration for 100% inhibition (μ g/ml)
HRP VI	17.8
HRP VIII	4.5
Heparin	150
RNase B	2.5
RNase B glycan	1.0
D2.11 anti-CSL	1.8
Fab anti-CSL	4.5
Fab anti-R1	> 215

Figure 6 Immunoblotting identification of CSL with human CDw70

A fraction specifically solubilized by mannose from young rat cerebella (see the Materials and methods section) was immunostained using D2.11 anti-CSL monoclonal antibody (lane 1), HNE51 anti-CDw70 monoclonal antibody (lane 2) and anti-CD69 monoclonal antibody (lane 3) as a control of specificity. Note that D2.11 and HNE51 stained the same protein subunits.

ligands [15], a similar role was expected for activated lymphocytes. This potential role of CSL in the aggregation of lymphocytes was analysed in an artificial system. Resting human lymphocytes (directly or after fixation with aldehyde mixtures) were agglutinated by the addition of CSL immunopurified from rat cerebellum or human lymphocytes (Figure 1b) at concentrations as low as 0.15–0.2 μ g/ml. As shown in Table 4, the calcium-independent agglutination (experiments were performed in the presence of 5 mM EDTA) induced by 1 μ g/ml CSL was specifically reversed by 4.5 μ g/ml of the anti-CSL Fab fragments and 1.8 μ g/ml of the D2.11 anti-CSL monoclonal antibody used in this study. It was also inhibited by oligomannosidic ligands of CSL (HRP isoenzyme VIII, 4.5 μ g/ml; RNase B, 2.5 μ g/ml; RNase B glycopeptide, 1.0 μ g/ml) and by heparin, 150 μ g/ml. The strong agglutinating activity of exogenously added CSL rendered credence to the hypothesis that CSL might participate in the aggregation of human lymphocytes observed after a 2 h stimulation with PMA.

Relationship between CSL and other 'activation-inducer molecules'

Other molecules considered as 'activation-inducer molecules'

appeared to have similar molecular masses to that of CSL: CD69, a C-type lectin [34,35], CD70 [36] and CDw70 [37]. Commercially available antibody to CD69 did not interact with CSL. In contrast, CSL and CDw70 were immunologically related, since the anti-CDw70 specifically identified the three molecular forms of CSL present in the rat cerebellum (31.5, 33 and 43 kDa), either in total homogenates or in immunopurified CSL preparations, as did the monoclonal and polyclonal anti-CSL antibodies. These protein bands were found only in a protein fraction specifically released with mannose from young rat cerebella (Figure 6). Other properties {kinetics of expression during activation; intracellular and extracellular accumulation in activated T- and B-cells, in activated neutrophils (J.-P. Zanetta, J. Wantyghem, S. Kuchler-Bopp, A. Badache and M. Aubery, unpublished work) and in malignant cells [15,38]; absence in platelets and red blood cells (J.-P. Zanetta, J. Wantyghem, S. Kuchler-Bopp, A. Badache and M. Aubery, unpublished work)} were comparable, suggesting that human CDw70 and rat CSL were related molecules.

DISCUSSION

The CSL molecule has been initially described as a calcium-independent, mannose-binding lectin endowed with a very fine discriminating property [39]. CSL binds to $\text{Man}_6\text{GlcNAc}_2$ oligomannosidic N-glycans, but not significantly to $\text{Man}_5\text{GlcNAc}_2$ and $\text{Man}_{7-9}\text{GlcNAc}_2$. This carbohydrate-binding specificity is close to that described for interleukin 2 [40]. CSL also binds to hybrid-type N-glycans having the HNK-1 epitope [15]. Its clustering properties made this lectin a potential candidate as an endogenous clustering agent essential for the generation of proliferation signal in human lymphocytes, as it does in other cells [15]. In order to test this hypothesis, we chose two well-documented experimental models of polyclonal lymphocyte activation. The one is the activation by mitogenic plant lectins like PHA and RPA [17], which are giving extracellular signals. The other is concerned with phorbol esters which are producing an intracellular signal by provoking the translocation of protein kinase C and also result in the activation of human lymphocytes.

Role of CSL in early phosphorylation–dephosphorylation mechanisms associated with lymphocyte activation

The phosphorylation–dephosphorylation switch-over (completed after a 2 h stimulation with PMA and a 24 h stimulation with plant lectins) is concerned with p56^{lck} and p59^{lyn} , which have been described as being associated with human lymphocyte activation [32,33]. This switch-over is largely CSL-dependent. Four sets of data support this view: (i) the kinetics of the switch-over in tyrosine phosphorylation are superimposable on those of CSL expression; (ii) this switch-over is abolished using relatively low amounts of anti-CSL Fab fragments; (iii) the addition of exogenous CSL to resting cells induces a rapid switch-over of major tyrosine-phosphorylated proteins; and (iv) phosphorylation occurring in resting cells is not modified by the addition of anti-CSL Fab fragments (the reason being that CSL is not expressed at the surface of resting cells). These Fab fragments are actually suppressing the carbohydrate-binding properties of CSL since they are also inhibiting the CSL-induced agglutination of fixed lymphocytes. The minor tyrosine-phosphorylated constituents (including doublets around 40 kDa and 120 kDa) which appear after a 24 h stimulation in the presence of PMA and only after a 72 h stimulation in the presence of RPA could correspond to previously described tyrosine-phosphorylated proteins [1–3,40–42]. These changes are also inhibited when cells stimu-

lated with PMA are cultured in the presence of anti-CSL Fab fragments.

The inhibitory effects of anti-CSL Fab fragments on the switch-over in the major tyrosine-phosphorylation associated with PMA and plant lectin stimulation and the action of exogenously added CSL indicate that the extracellular CSL plays an important role, although the major quantitative increase of CSL occurs intracellularly after stimulation with PMA and plant lectins. The predominant role of the externalized CSL was also deduced from studies on the developing nervous tissue [13,15]. The mechanism suggested by this study is that the PMA-induced translocation of protein kinase C results in the expression of CSL as an early event (about a 10-fold increase within 2 h). This increase is followed by significant externalization and binding to surface ligands. The subsequent clustering governs the switch-over in major phosphorylation–dephosphorylation mechanisms. In the case of the PHA and RPA, the first events are probably PHA- and RPA-dependent clustering of the ligands of the plant lectins. This clustering did not induce the major changes of tyrosine-phosphorylations observed with CSL. Probably, another pathway is stimulated [33], inducing a weak signal for CSL synthesis. Progressively, following the synthesis of CSL and its expression at the surface, a CSL-dependent clustering begins, and specific CSL-dependent phosphorylations. The competition between stimulations by plant lectins and PMA (observed in Table 1) may find explanation in the competition of CSL and plant lectins for the same ligands in the first hours of the activation process [RPA (or ConA) binds to glycoproteins having the same molecular mass as the CSL ligands]. Our results suggest that, regardless of the initial activation signal, a second process begins when CSL is fully expressed at the lymphocyte surface for ensuring the clustering of its ligands. Since the quantity of CSL ligands is constant, the efficiency of the clustering depends only upon the CSL concentration at the surface, which reaches a plateau. Thus, CSL behaves as a 'saturable signal amplifier' necessary for establishing the complete switch-over in tyrosine phosphorylation between p56^{lck} and p59^{lyn} . This would induce the proper signal for expression of oncogene products and receptors necessary for the activation process.

New insights invoked by the role of CSL

The nature of the CSL ligands give credence to the role of CSL as an endogenous clustering molecule important for the phosphorylations–dephosphorylations occurring in the early stages of lymphocyte activation and for maintaining the activation signal. In T-cells, one of the CSL ligands is CD3, which is part of the TCR complex. The CSL may be the endogenous effector of the activation observed artificially using the anti-CD3 monoclonal antibody, which also induces a clustering of TCR molecules. However, since other CSL ligands are present in T-cells (in particular the low-molecular-mass glycoproteins), it is suggested that CSL can also associate TCR complexes with other surface molecules. Unfortunately, the nature of these molecules is unknown, although some of them may correspond to GPI-anchored B7 glycoproteins [27], which were shown to induce a co-stimulatory effect on the CD3-dependent activation of T-lymphocytes [41]. In B-cells, one of the CSL ligands is the GPI-anchored CD24 which is involved in B-cell activation [26]. The very peculiar structure of this molecule (a very short polypeptide chain of about 30 amino acids endowed with three N-glycans) favours the role of its carbohydrate moiety. CSL may serve for clustering CD24 molecules and for associating CD24 with the other CSL ligands, especially the low-molecular-mass glycoproteins.

The presence of a lectin like CSL can explain several experimental observations concerned with the mechanism of lymphocyte activation. The differential effects of inhibitors of N-glycan processing on T-cell activation induced by ConA [9] suggest the presence of a clustering agent for TCR having similar carbohydrate-binding properties to CSL. The initial signal triggered by ConA [33] is relayed by an endogenous polyvalent carbohydrate-binding protein, such as CSL, capable of recognizing hybrid-type N-glycans produced in the presence of swainsonine. Consequently, bridging TCR molecules can occur. In contrast, although the initial signal triggered by ConA can occur in cells treated with castanospermine, the true signal cannot occur because CSL does not recognize glucosyl-oligomannosidic N-glycans.

Another example is concerned with the cross-activation of quiescent lymphocytes by activated cells, independent of cytokines or antigens [42,43]. Activated T-cells express surface CSL, which is involved in clustering of its ligands, but still has free binding sites capable of interacting with unoccupied N-glycans at the surface of quiescent T- or B-cells. This adhesion initiates marked clustering of ligands at the surface of quiescent cells and, consequently, activation through CSL-dependent phosphorylations-dephosphorylations. The putative role of CSL in the aggregation of activated lymphocytes is supported by the strong calcium-independent, carbohydrate-dependent, agglutination of resting lymphocytes using exogenous CSL. The involvement of surface N-glycans in cross-activation is supported by the inefficiency of tunicamycin-treated cells in activating lymphocytes in the mixed lymphocyte reaction [44]. Furthermore, the role of a lectin with a carbohydrate-binding property similar to that of CSL in the cross-activation mechanism between T- and B-cells is strongly supported by the finding that such activation is dependent upon mannose-rich N-glycans [43]. Although N-glycosylation inhibitors like tunicamycin and castanospermine can inhibit most of the lectin-dependent processes of activation, the positive actions of swainsonine and deoxymannojirimycin favour a role of lectins having such a specificity. Thus, the early increased homotypic and heterotypic adhesiveness of activated lymphocytes (T-T, T-B, T- and B-neutrophils) frequently observed, may find explanation by considering the role of CSL.

Relationship between CSL and identified molecules

The question remains as to whether CSL corresponds to already described 'activation-inducer molecules'. CD70 (CD27 ligand) binds to CD27 [45], and is considered as important for lymphocyte activation and adhesion. However, CD70 is a membrane-bound glycoprotein. The time-course of expression of CSL and CD70 are also different during activation (48 h difference for reaching maximal levels). In contrast CSL and CDw70 [37] are not distinguishable for their cellular specificity, localization and kinetics of expression. Furthermore, the commercially available monoclonal antibody against the human CDw70 recognizes all rat CSL subunits. Thus, it is evident that the human CDw70, defined by the monoclonal antibody HNE51 (Immunotech) [37], corresponds to the rat CSL (defined by the D2.11 monoclonal antibody and its polyclonal antibody), but is markedly different from the CD70/CD27 ligand, defined by the 2F2 antibody [45]. The cDNA sequence of rat CSL (J.-P. Zanetta, J. Wantyghem, S. Kuchler-Bopp, A. Badache and M. Aubery, unpublished work) supports this view.

We thank J.-C. Barthe, J.-G. Lorentz, A. Meyer and M. Zaeffel for technical assistance. This work was supported by grants from the National Multiple Sclerosis Society and the Association pour la Recherche contre le Cancer.

REFERENCES

- Lipfert, L., Haimovich, B., Schaller, M. D., Cobb, B. S., Parsons, J. T. and Brugge, J. S. (1992) *J. Cell Biol.* **119**, 905–912
- Kornberg, L. J., Earp, H. S., Turner, C. E., Prockop, C. and Juliano, R. L. (1991) *Proc. Natl. Acad. Sci. U.S.A.* **88**, 8392–8396
- Kornberg, L., Earp, H. S., Parsons, J. T., Schaller, M. and Juliano, R. L. (1992) *J. Biol. Chem.* **267**, 23439–23442
- Beyers, A. D., Spruyt, L. L. and Williams, A. F. (1992) *Trends Cell Biol.* **2**, 253–255
- Izquierdo, M. and Cantrell, D. A. (1992) *Trends Cell Biol.* **2**, 268–271
- Davies, M. P. A., Hallam, T. J. and Merritt, J. E. (1990) *Biochem. J.* **267**, 13–16
- Elenström-Magnusson, C., Altevogt, P. and Severinson, E. (1993) *Eur. J. Immunol.* **23**, 888–892
- Feizi, T. and Childs, R. A. (1987) *Biochem. J.* **245**, 1–11
- Bowlin, T. L., Schroeder, K. K. and Fanger, B. O. (1991) *Cell. Immunol.* **137**, 111–117
- Zanetta, J.-P., Meyer, A., Kuchler, S. and Vincendon, G. (1987) *J. Neurochem.* **49**, 1250–1257
- Zanetta, J.-P., Kuchler, S., Lehmann, S. et al. (1992) *Histochem. J.* **24**, 791–804
- Fressinaud, C., Kuchler, S., Sarliève, L. L., Vincendon, G. and Zanetta, J.-P. (1988) *C. R. Acad. Sci. Paris, Série III*, **307**, 863–868
- Lehmann, S., Kuchler, S., Théveniau, M., Vincendon, G. and Zanetta, J.-P. (1990) *Proc. Natl. Acad. Sci. U.S.A.* **87**, 6455–6459
- Lehmann, S., Kuchler, S., Badache, A., Zaeffel, M., Meyer, A. and Zanetta, J.-P. (1991) *Eur. J. Cell Biol.* **56**, 433–442
- Zanetta, J.-P., Badache, A., Maschke, S., Marschal, P. and Kuchler, S. (1994) *Histol. Histopathol.* **9**, 385–412
- Carrera, A. C., Sanchez-Madrid, F., Lopez-Botet, M., Bernabeu, C. and de Landazuri, M. O. (1987) *Eur. J. Immunol.* **17**, 179–184
- Wantyghem, J., Goulut, C., Frénoy, J.-P., Turpin, E. and Goussault, Y. (1986) *Biochem. J.* **237**, 483–489
- Zanetta, J.-P., Dontenwill, M., Meyer, A. and Roussel, G. (1985) *Dev. Brain Res.* **17**, 233–243
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Towbin, H., Staehelin, T. and Gordon, J. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 4350–4354
- Leary, J. J., Brigati, D. J. and Ward, D. C. (1983) *Proc. Natl. Acad. Sci. U.S.A.* **80**, 4045–4049
- Zanetta, J.-P., Maschke, S. and Badache, A. (1993) in *Lectins and Glycobiology* (Gabijs, H.-J. and Gabijs, S., eds.), pp. 182–187, Springer Verlag, Heidelberg
- Glass, W. F., II, Briggs, R. C. and Hnilica, L. S. (1981) *Anal. Biochem.* **115**, 219–224
- Graham, R. C. and Karnovsky, M. J. (1966) *J. Histochem. Cytochem.* **14**, 291–302
- Bierer, B. E. and Burakoff, S. J. (1991) *Adv. Cancer Res.* **56**, 49–76
- Kay, R., Rosten, P. M. and Humphries, R. K. (1991) *J. Immunol.* **147**, 1412–1416
- Nédelec, J., Pierres, M., Moreau, H., Barbet, J., Naquet, P., Faivre-Sarrailh, C. and Rougon, G. (1992) *Eur. J. Biochem.* **203**, 433–442
- Kuchler, S., Rougon, G., Marschal, P., Lehmann, S., Reeber, A., Vincendon, G. and Zanetta, J.-P. (1989) *Neuroscience* **33**, 111–124
- Altman, A., Mustelin, T. and Coggeshall, K. M. (1990) *Crit. Rev. Immunol.* **10**, 347–391
- Burridge, K., Turner, C. E. and Romer, L. H. (1992) *J. Cell Biol.* **119**, 893–903
- Kohno, M., Chatani, Y., Tanaka, E., Hattori, A. and Nishizawa, N. (1992) *Biochem. J.* **287**, 917–924
- Bohuslav, J., Cinek, T. and Horejsi, V. (1993) *Eur. J. Immunol.* **23**, 825–831
- Takeuchi, F., Taniguchi, T., Maeda, H., Fujii, C., Takeuchi, N. and Yamamura, H. (1993) *Biochem. Biophys. Res. Commun.* **194**, 91–96
- Cebrian, M., Yagüe, E., Rincon, M., Lopez-Botet, M., de Landazuri, M. O. and Sanchez-Madrid, F. (1988) *J. Exp. Med.* **168**, 1621–1637
- Ziegler, S. F., Levin, S. D., Johnson, L. et al. (1994) *J. Immunol.* **152**, 1228–1236
- Goodwin, R. G., Alderson, M. R., Smith, C. A. et al. (1993) *Cell* **73**, 447–487
- Al Saati, T., Mazerolles, C., Caspar, S. et al. (1989) in *Leukocyte Typing IV. White Cell Differentiation Antigens* (Knapp, W., et al., eds.), pp. 452–455, Oxford University Press, Oxford, New York, Tokyo
- Maschke, S., Robert, J., Coindre, J.-M., Kuchler, S., Vincendon, G. and Zanetta, J.-P. (1993) *Eur. J. Cell Biol.* **62**, 163–172
- Marschal, P., Reeber, A., Neeser, J.-R., Vincendon, G. and Zanetta, J.-P. (1989) *Biochimie* **71**, 645–653
- Sherblom A. P., Sathyanarayanan, N., Decker, J. M. and Muchmore, A. V. (1989) *J. Immunol.* **143**, 939–944
- Stikakis, P. P., Ogleby, R., Stikakis, P. and Tsokos, G. C. (1994) *Clin. Exp. Immunol.* **96**, 8–14
- Chirmule, N., Kalyanaraman, V. S., Lederman, S. et al. (1993) *J. Immunol.* **150**, 2478–2486
- Savage, S. M., Donaldson, L. A. and Sopori, M. L. (1993) *Cell. Immunol.* **146**, 11–27
- Hart, G. W. (1982) *J. Biol. Chem.* **257**, 151–158
- Hintzen, R. Q., Lens, M. S., Beckmann, M. P., Lynch, D., Goodwin, R. G. and van Lier, R. A. W. (1994) *J. Immunol.* **152**, 1762–1773