Phosphorylation of Oct-2 at sites located in the POU domain induces differential down-regulation of Oct-2 DNA-binding ability

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We compared the effects of phosphorylation of Oct-2 protein on its binding to the consensus octamer sequence (ATGCAAAT) and two non-canonical sequences present in human (AAGCAAT) and murine (AAACAAAT) promoters of the *BLR1* (Burkitts' lymphoma receptor 1) gene encoding chemokine receptor CXCR5 (CXC-chemokine receptor 5). The latter *cis*-acting elements represent low-affinity recognition sequences for the octamer transcription factors. Okadaic acid was found to induce hyperphosphorylation of Oct-2 specifically in cells of lymphoid lineage. Potentially phosphorylated amino acid residues localized to the POU-specific domain of Oct-2. Whereas binding of Oct-2 to the octamer site from the human BLR1 promoter or to the consensus octamer sequence was unaffected

by phosphorylation of this factor, a strong reduction of Oct-2 binding to the octamer site from the murine BLR1 promoter was observed. This finding correlates well with the down-regulation of expression of the *BLR1* gene in murine splenic cells but not in lymphoid cells of human origin treated with okadaic acid. These data support the hypothesis that phosphorylation of Oct-2 may be a mechanism by which activities of the promoters containing non-canonical octamer sequences are differentially regulated in response to extracellular stimuli.

Key words: CXCR5, gene expression, octamer sequence, okadaic acid.

INTRODUCTION

The consensus octamer motif 5'-ATGCAAAT-3', which is present in the promoters of genes displaying a widespread as well as a tissue-specific expression pattern, is a recognition site of the ubiquitously expressed Oct-1 [1,2] and the B-cell-restricted Oct-2 [3–5] transcription factors. These two proteins belong to the POU family of transcription factors, including additionally the mammalian Pit-1 factor and the developmental control protein unc86 of *Caenorhabditis elegans*, hence the name of this group (Pit, Oct, Unc). All these factors share the conserved POU domain of approx. 160 amino acids in length, consisting of an N-terminal POU-specific sequence and a homeodomain that is closely related to other homeobox proteins. Both subdomains are connected by a short variable linker sequence. The entire POU domain is required for sequence-specific DNA binding and also mediates protein–protein interactions (for reviews see [6–8]).

Regulation of gene expression by post-translational modifications of transcription factors is a commonly observed mechanism. Phosphorylation and dephosphorylation of transcription factors are the most frequently used mechanisms, which regulate their activity in response to different extra- and intracellular signals and enable convergence of different signalling pathways at the same factor. Phosphorylation may cause diverse effects on transcription factor activity and affect translocation to the nucleus, DNA binding or interaction of a transcription factor with the basal transcription apparatus (reviewed in [9,10]). Activities of several members of the POU-domain factors' family were shown to be modulated by this mechanism. Oct-1 is hyperphosphorylated as cells enter mitosis and this correlates with a strongly reduced binding of this factor to the octamer site and a concomitant inhibition of transcription. Phosphorylation

of Oct-1 is rapidly reversed as cells exit mitosis and enter the G₁ phase of the cell cycle [11,12]. It was shown that mitosis-specific phosphorylation of a single serine residue located in the homeodomain of Oct-1 by cAMP-dependent protein kinase A was sufficient to inhibit DNA-binding ability of this factor [13]. Another member of the POU-domain family, Pit-1, was shown to become phosphorylated in pituitary cells at two distinct sites in the homeodomain in response to phorbol esters and cAMP [14].

Although there are many indications that changes of the phosphorylation status of the POU-domain proteins modulate their ability to activate transcription, very little is known about the role phosphorylation plays in the transcriptional potential of the Oct-2 factor. Initial observations indicated that Oct-2 is phosphorylated *in vivo* and suggested that this factor becomes more competent to activate transcription in the phosphorylated state [15]. However, the location of the sites of phosphorylation in the Oct-2 factor were not identified in this study. Based on the observed increase in the activation potential of the phosphorylated Oct-2 it was suggested that phosphorylation causes conformational changes of the protein that either create a new or unmask an existing activation domain. In another study the effects of different kinases and inhibitors of phosphatases on the DNA binding of nuclear Oct-2 were investigated *in vitro* [16]. This study revealed that both tested kinase activities and inhibition of cellular phosphatases modulated Oct-2 DNA binding in a binding-site-dependent mode. In particular, inhibition *in vitro* of protein phosphatase 1 (PP1) and PP2A present in Daudi cell nuclear extracts by okadaic acid produced no effect on the Oct-2 binding to the TAATGARAT motif from Herpes simplex virus immediate early 4/5 genes. At the same time okadaic acid added to the nuclear extracts effectively inhibited binding of Oct-2 to

Abbreviations used: BLR1, Burkitts' lymphoma receptor 1; CK II, casein kinase II; CXCR5, CXC-chemokine receptor 5; DTT, dithiothreitol; EMSA, electrophoretic mobility-shift assay; GADPH, glyceraldehyde-3-phosphate dehydrogenase; GST, glutathione S-transferase; PKA, protein kinase A; PP, protein phosphatase.

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the classical octamer sequence derived from the histone 2B gene promoter [16]. Therefore, this study [16] indicated that phosphorylation could represent a specific mechanism whereby Oct-2 binding to low-affinity DNA binding sites would be regulated independently of high-affinity sites.

BLR1/CXCR5 (Burkitt's lymphoma receptor 1/CXC-chemokine receptor 5) is a lymphoid-specific chemokine receptor that directs migration of lymphocytes within specific compartments of the immune system [17,18]. Expression of the receptor BLR1 is restricted to mature resting B cells and to a subpopulation of T-helper cells [19]. The promoter sequences of the human and murine *BLR1* genes are highly homologous and the identified binding sites are almost identical in both promoters [20,21]. We demonstrated previously that the expression of the *BLR1* gene is regulated in both humans and mice in an Oct-2 factor-dependent mode. The non-canonical octamer motif located downstream of the transcription start site was identified as the most essential promoter element, which mediates activation of the promoter by Oct-2 in both species. A mutation that effectively blocked binding of octamer factors to this site resulted in a complete inactivation of the promoter activity [20]. In the present study we compared the ability of hyperphosphorylated Oct-2 to bind to the octamer motif 5'-AAGCAAAT-3' of the human BLR1 promoter sequence and to the motif 5'-AAACAAAT-3' of the murine sequence. We show that phosphorylation of this factor *in vivo* results in a selective down-regulation of its binding to the low-affinity octamer sequence of the murine but not of the human BLR1 promoter. This observation was found to correlate with the abrogation of the *BLR1* gene expression in murine cells, suggesting that phosphorylation of Oct-2 may exert profound effects on BLR1 expression in mice.

MATERIALS AND METHODS

Cell culture, transient transfection and reporter assay

The single-cell suspensions of mononuclear cells from human tonsils or murine spleens were obtained by disruption of the freshly harvested organs through a fine stainless steel sieve into ice-cold PBS supplemented with 3% fetal calf serum. The red blood cells were depleted from the suspension by lysis in hypotonic ammonium chloride solution. The primary cell cultures as well as the haematopoietic cell lines (Raji, Namalwa, Abl/Oct2-ER) were cultivated in RPMI 1640 medium supplemented with 10% (v/v) fetal calf serum at 37 °C with 5% CO₂. The exponentially growing cells were transfected by electroporation (1070 µF and 250–300 V) with 20–40 µg of plasmid DNA, harvested 24 h after transfection and lysed with reporter lysis buffer (Promega, Madison, WI, U.S.A.). Luciferase activity was determined in an LB 9501 luminometer (EG+G Berthold, Bad Wildbad, Germany) using a luciferase assay system (Promega).

Plasmid constructs and site-directed mutagenesis

The pOEVI vector expressing human Oct-2 was kindly provided by W. Schaffner (University of Zurich, Zurich, Switzerland). The constructs expressing mutated Oct-2 protein were generated by PCR-based site-directed mutagenesis of the fragment corresponding to the open reading frame of Oct-2 with subsequent cloning into the pcDNA3.1 vector (Invitrogen, Carlsbad, CA, U.S.A.). The construct blr1MH-Luc was generated using the QuikChange[™] site-directed mutagenesis kit (Stratagene, La Jolla, CA, U.S.A.). All mutated constructs were controlled by

sequencing. To generate the pGEX-Oct2 construct, the Oct-2 open-reading-frame fragment was cloned in the pGEX-2T vector (Pharmacia, Piscataway, NJ, U.S.A.) in frame with the coding region of the glutathione S-transferase (GST) domain.

Preparation of protein extracts

Nuclear extracts from Raji cells were prepared as described by Schreiber et al. [22]. To prepare whole-cell extracts, 10⁶–10⁷ harvested cells were washed with an ice-cold buffered saline solution, collected by centrifugation and resuspended in 50–200 µl of an ice-cold high-salt buffer [20 mM Hepes (pH 7.9), 0.35 M NaCl, 1 mM MgCl₂, 0.2 mM EDTA, 0.1 mM EGTA, 1% Nonidet P-40, 20% glycerol, 1 mM dithiothreitol (DTT), 5 µg/ml aprotinin, 5 µg/ml leupeptin, 5 µg/ml pepstatin A]. The suspension was vigorously vortexed for 30 s and was shaken for 20 min at 4 °C on a shaking platform. The extract was cleared by centrifugation (13800 g at 4 °C for 5 min) and protein concentrations were determined by the Bradford method (Bio-Rad Laboratories GmbH, Munich, Germany). The extracts were either used for further analysis immediately after preparation or aliquotted and stored at –80 °C.

Electrophoretic mobility shift assays (EMSAs)

EMSAs were performed with the double-stranded oligonucleotides labelled by filling in the overhangs with *Taq* polymerase (Gibco-BRL) in the presence of radioactive dNTPs at 54 °C. The sequences of the oligonucleotides used (top strand only) were as follows: mur-Oct 5'-GGATGGCTC-ATTGTTTAAGTT; hum-Oct 5'-GGAGCTCATTGCTT-AAA; and Oct-wt 5'-GGCAGAAAGCTCATTGTCATTA-AGACC (where the underlined sequence indicates the octamer-binding site).

Approx. 5–10 µg of nuclear or whole-cell extracts was incubated with 30 fmol of labelled oligonucleotides in a binding buffer [10 mM Hepes (pH 7.9), 0.1 M KCl, 1 mM EDTA, 1 mM DTT, 4% ficoll] containing 50 µg/ml double-stranded poly(dI-dC) (Pharmacia Biotech, Uppsala, Sweden) for 15 min at room temperature. Subsequently the reaction mixtures were separated in a 5% (w/v) polyacrylamide gel in 0.5×TBE (Tris/borate/EDTA) buffer. After electrophoresis the gel was dried and autoradiographed. Alternatively the retarded complexes were visualized with Fuji BAS 1000 bioimaging plates for quantification of the results.

Western-blot analysis

Probes of nuclear or whole-cell extracts containing 20 µg of protein were heated at 95 °C for 5 min and resolved by electrophoresis through SDS/polyacrylamide (10%) gels [23]. Proteins were electrophoretically transferred on to nitrocellulose (Hybond-C, Amersham) and immunoblotting was performed in TBST [10 mM Tris/HCl (pH 8.0), 150 mM NaCl and 0.05% Tween 20]/5% non-fat dry milk powder using the rabbit polyclonal anti-(human Oct-2) antibody (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) at 0.1 µg/ml and alkaline phosphatase-conjugated secondary antibody (Sigma) at 1:2000 dilution. Immunoreactive proteins were visualized with the chromogenic reaction substrate NBT/BCIP (Nitro Blue Tetrazolium/5-bromo-4-chloroindol-3-yl phosphate; Promega).

RNA isolation, Northern blotting and hybridization

Total RNA was prepared with the TRIzol reagent (Gibco-BRL) according to the supplied protocol. Total RNA (30 µg per lane)

was resolved through a denaturing 1.2% agarose gel and transferred on to a nylon membrane (Hybond-N, Amersham) according to the previously described procedure [24]. For hybridization the filter was incubated for 2 h at 68 °C with hybridization buffer [0.5 M Na₂HPO₄ (pH 7.2), 7% SDS, 1 mM EDTA and 1% BSA] after which the radiolabelled probe was added and the filter incubated at 68 °C for 24 h. The filter was washed with 2 × SSC/0.1% SDS (where 1 × SSC is 0.15 M NaCl/0.015 M sodium citrate) twice for 20 min at room temperature followed by stringent washing steps with 0.2 × SSC/0.1% SDS at 55 °C. The blot was exposed to PhosphorImaging plates and the image read with the bioimaging analyser (Fujix BAS 2000). The absorbances of the bands were determined using the bioimaging analysis software (MacBas).

Expression and phosphorylation *in vitro* of the recombinant Oct-2 protein

Escherichia coli were transformed with the construct pGEX-Oct2 and induced with isopropyl β-D-thiogalactoside according to the standard protocol. Cells were harvested and subjected to French-press lysis in suspension buffer (150 mM NaCl, 16 mM Na₂HPO₄, 4 mM NaH₂PO₄ and 1% Triton X-100). The cleared lysate was applied to a column of glutathione-agarose. The column was washed extensively with the suspension buffer after which the matrix particles with the bound GST fusion proteins were washed and resuspended in 50 mM Tris/HCl (pH 8.0).

Phosphorylation *in vitro* of the bound proteins was performed with crude Raji cell lysates. Exponentially growing cells were treated for 2 h with 100 ng/ml okadaic acid (Alexis Corporation, Läufelfingen, Switzerland) and resuspended in ice-cold lysis buffer [20 mM Tris/acetate (pH 7.0), 0.1 mM EDTA, 1 mM EGTA, 1 mM Na₃VO₄, 10 mM β-glycerophosphate, 50 mM NaF, 5 mM pyrophosphate, 1% Triton X-100, 0.27 M sucrose, 2 mM DTT, 5 μg/ml aprotinin, 5 μg/ml pepstatin A, 5 μg/ml leupeptin and 100 ng/ml okadaic acid] at a concentration of 10⁶ cells per 100 μl. The suspension was vortexed vigorously for 30 s, incubated on ice and clarified by centrifugation (13800 g at 4 °C for 5 min). The suspension of agarose matrix with the bound fusion proteins was mixed with 100–200 μg of the cell lysate in the reaction buffer (50 mM sodium β-glycerophosphate, 0.1 mM EDTA, 4 mM magnesium acetate and 0.1 mM ATP) containing 5 μCi of [γ-³²P]dATP (NEN Life Science Products, Bad Homburg, Germany) per reaction. The reaction mix was incubated at 30 °C for 1 h. The agarose matrix was precipitated by brief centrifugation at 320 g and washed 5–8 times in suspension buffer. After the final washing step the GST fusion proteins were eluted from agarose into Laemmli loading buffer by heating at 95 °C for 5 min. The proteins were separated in a SDS/polyacrylamide (10%) gel. After the electrophoretic separation the gel was dried and exposed to a Fuji BAS 1000 bioimaging plate for visualization of labelled proteins.

Proteolytic phosphopeptide analysis of Oct-2 protein

Gel bands producing the strongest radioactive signal were excised from the gel with a scalpel, cut into small gel cubes, washed extensively in 0.1 M Tris/acetonitrile (1:1, v/v) and dried under vacuum. After reduction and alkylation *in situ* by iodoacetamide, proteolytic digestion of gel-immobilized proteins was performed overnight at 37 °C with either 0.5 μg of trypsin or 1 μg of chymotrypsin (Boehringer Mannheim, Mannheim, Germany) in 150 μl of 0.1 M Tris/HCl buffer (pH 8.1). The peptides were recovered from the gel pieces by 3-fold extraction with a mixture of 60% acetonitrile/0.1% trifluoroacetic acid at 30 °C for

20 min. After reducing the volume of eluent to approx. one-quarter in a Speed Vac, the eluted peptides were fractionated by reverse-phase HPLC, applying the mixture to a μRPC C₂/C₈SC2.1/10 narrow-bore column from the SMART[®] system (100 × 2.1 mm; LKB Pharmacia, Uppsala, Sweden). The peptides were eluted with 0.1% trifluoroacetic acid buffer using a gradient of increasing concentration of acetonitrile (5–50%). Approx. 80 fractions were collected automatically and subjected to Cerenkov radioactivity counting (LS 6000LL, Beckman Instruments) in duplicate measurements. Peptide identification of fractions that produced a radioactive signal significantly exceeding the background level was performed with a Procise Protein Microsequencer (Applied Biosystems, Foster City, CA, U.S.A.). In some cases peptide size was determined by MS (Q-tof, Micro-mass, Manchester, U.K.).

RESULTS

Okadaic acid induces hyperphosphorylation of cellular Oct-2 on serine and/or threonine residues

To investigate the effect of phosphorylation of Oct-2 on the functional activity of this factor we incubated Raji cells for 2 h at 37 °C with 100 ng/ml (0.125 μM) okadaic acid. This substance is a strong tumour promoter that acts by inhibiting the cellular serine/threonine-specific PP1 and PP2A and by inducing phosphorylation of numerous cellular proteins [25]. The nuclear extracts from cells stimulated by okadaic acid as well as from unstimulated cells containing equal amounts of protein were analysed by immunoblotting using a polyclonal Oct-2-specific antibody. In unstimulated cells (Figure 1A, lane 1) Oct-2 is represented by a heterogeneous array of species characterized by different mobilities in SDS/PAGE. Inhibition of cellular phosphatases by okadaic acid results in a decrease of the electrophoretic mobility of all species (Figure 1A, lane 2). The observed effect of okadaic acid could be completely abrogated by a 2-h preincubation of cells with 0.5 μM staurosporine (Sigma), which is an inhibitor of a broad spectrum of kinases (Figure 1A, lane 3). However, none of the tested specific inhibitors of protein kinase C [Ro-31-8220 (Calbiochem, San Diego, CA, U.S.A.); 1 μM], phosphatidylinositol 3-kinase and DNA-dependent protein kinase [wortmannin (Sigma); 0.5–4 μM] or protein kinase A [PKA; KT5720 (Calbiochem); 0.5 μM] could block the phosphorylation of Oct-2 (results not shown).

Figure 1 Electrophoretic heterogeneity of Oct-2 in Raji cells is determined by phosphorylation of serine and/or threonine residues

Oct-2 was detected specifically on Western blots produced with nuclear extracts from Raji cells treated in culture as follows. (A) Unstimulated cells (lane 1), cells stimulated for 2 h by 100 ng/ml okadaic acid (lane 2) or cells incubated for 2 h with 0.5 μM staurosporine prior to stimulation by okadaic acid (lane 3). (B) Nuclear extracts from cells stimulated by okadaic acid (lane 1) were incubated at 37 °C for 30 min with 20 units of alkaline phosphatase (lane 2) or with tyrosine-specific YOP phosphatase (lane 3).

Figure 2 Comparison of the abilities of Oct-2 and Oct-1 to bind to the classical octamer sequence and to the non-canonical octamer sites of the human and murine *BLR1* promoters

EMSA was performed with the labelled Oct-wt oligonucleotide incubated with nuclear extract prepared from Raji cells. The complexes were competed either by excess of unlabelled Oct-wt oligonucleotide or by hum-Oct or mur-Oct oligonucleotides as indicated; ns, non-specific. The bold letters in the octamer-binding sequences indicate the differences between the canonical sequence and the non-canonical sequences present in the promoters of the human and the murine *BLR1* genes.

As hyperphosphorylation of Oct-2 upon inhibition of serine/threonine-specific phosphatases may be a result of activation of cellular kinases which are regulated by phosphorylation itself, we next tested whether Oct-2 is phosphorylated on tyrosine residues. Treatment of the nuclear extracts from cells stimulated by okadaic acid with unspecific alkaline phosphatase (Figure 1B, lane 2) as well as with serine/threonine-specific phosphatase PP1 (results not shown) effectively reduced the level of Oct-2 phosphorylation. In contrast, treatment of nuclear extracts with the tyrosine-specific phosphatase YOP (Figure 1B, lane 3) did not alter the pattern of electrophoretic mobility of the Oct-2 species present in the extracts.

Interestingly, stimulation of HeLa or HEK 293 cells transiently transfected with Oct-2 expression construct pOEVI by okadaic acid did not result in hyperphosphorylation of ectopically expressed Oct-2 protein (results not shown). Although the identity of the kinase or kinases involved in Oct-2 phosphorylation remains obscure, they are likely to be either specifically expressed in lymphoid cells or inactive in non-lymphoid cells under the conditions of the experiments.

Phosphorylation of Oct-2 differentially reduces protein binding to the non-canonical octamer sequence of the murine *BLR1* gene promoter

We next tested whether phosphorylation of Oct-2 influences its ability to bind to the non-canonical octamer sites present in the human and murine *BLR1* gene promoters. Both Oct-1 and Oct-2 factors bind to these motifs with a lower affinity than to the consensus octamer site (Figure 2). Treatment of Raji cells with okadaic acid dramatically reduced the ability of Oct-2 and also of Oct-1 present in nuclear extracts from these cells to bind to the oligonucleotide mur-Oct, corresponding to the octamer site of the murine *BLR1* promoter (Figure 3A, lanes 1 and 2). In

Figure 3 Comparison of the ability of hyperphosphorylated Oct-2 and Oct-1 to bind different octamer sequences

(A) EMSA was performed with oligonucleotides mur-Oct, hum-Oct and Oct-wt and nuclear extracts from untreated Raji cells (lanes 1, 3 and 5) and cells stimulated with okadaic acid (lanes 2, 4 and 6). (B) Relative binding abilities of Oct-2 and Oct-1 with three tested oligonucleotides. Relative binding ability was calculated as the ratio of absorbances of the bands formed by binding of Oct-2/Oct-1 from cells treated with okadaic acid to the bands formed by Oct-2/Oct-1 from untreated cells. The bars represent the means $\pm$ S.D. of relative binding abilities determined in four independent experiments.

contrast, incubation of the same nuclear extracts with the oligonucleotides corresponding to either the octamer sequence of the human *BLR1* promoter (hum-Oct; Figure 3A, lanes 3 and 4) or the consensus octamer sequence (Oct-wt; Figure 3A, lanes 5 and 6) showed that Oct-2 binds to both these sites almost equally well as it does prior to hyperphosphorylation (Figure 3B), thus excluding the possibility that treatment of cells with okadaic acid reduces the amount of Oct-2 factor. In addition, immunoblotting analysis showed that treatment of Raji cells with okadaic acid does not significantly influence the amount of Oct-2 protein present in the nuclear extracts (see also Figure 1). At the same time Oct-1 binding to both oligonucleotides hum-Oct and Oct-wt was significantly reduced upon stimulation of cells by okadaic acid (Figure 3). The specific impairment of Oct-2 binding to the octamer site of the murine *BLR1* promoter could be efficiently prevented when the cells were incubated with 0.5 μ M staurosporine for 2 h prior to the stimulation by okadaic acid (results not shown), providing additional evidence that the observed effect is due to the phosphorylation of this factor.

Okadaic acid blocks the expression of the *BLR1* gene in murine splenocytes but not in human B cells

In the promoters of both human and murine *BLR1* genes the non-canonical octamer site is an essential regulatory element and

Figure 4 Influence of okadaic acid treatment on the expression of *BLR1* gene in murine and human B lymphocytes

(A) Cells were incubated with 100 ng/ml okadaic acid for various periods of time as indicated. Total cellular RNA (30 µg per lane) was analysed by Northern-blot hybridization with the human *BLR1* gene cDNA and the human *GAPDH* gene cDNA probes. Representative data are shown for splenocytes isolated from Balb/c mice and Raji cells only. (B) Densitometric analysis of the Northern blots including data obtained with tonsillar cells. Relative absorbance (optical density) was calculated as the ratio of the amount of the hybridized *BLR1* mRNA to the amount of the *GAPDH* mRNA for each incubation time point. The values obtained with the control (0 h) were set to 1 in all experiments and all other values are shown relative to the control.

the binding of Oct-2 to this element plays a critical role in the regulation of promoter activity [20]. As described above, hyperphosphorylation of Oct-2 strongly reduces binding of this factor to the octamer site of the murine promoter but leaves the binding to the octamer site of the human gene promoter almost unaffected. It was interesting to test whether down-regulation of Oct-2 binding to the promoter sequence correlates with a down-regulation of *BLR1* gene expression. For this purpose mononuclear cell suspensions were prepared from spleens of Balb/c mice and incubated in culture with 100 ng/ml okadaic acid for 2, 4 and 8 h. In parallel, Raji cells or primary tonsillar mononuclear cells were treated with okadaic acid for the same periods of time. The steady-state levels of *BLR1* mRNA isolated from these cell cultures were visualized by Northern-blot analysis using a cDNA probe of the human *BLR1* gene. The amount of *BLR1*-specific mRNA was quantified by normalization with respect to *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase) gene expression. As shown in Figure 4, treatment of the murine cells with okadaic acid almost completely down-regulated the expression of the *BLR1* gene, whereas the expression of this gene in cells of human origin was unaffected by stimulation with okadaic acid for up to 8 h.

To substantiate the assumption that down-regulation of Oct-2 binding to the murine *BLR1* promoter results in the inactivation of its function, we tested further if okadaic acid affects the expression of the luciferase gene from the reporter construct containing the promoter of the murine *BLR1* gene. For this purpose, Namalwa cells were transfected with the blr1-Luc luciferase reporter construct [20], and were divided into three

Figure 5 Influence of okadaic acid treatment on the expression of luciferase from the blr1-Luc and blr1MH-Luc reporter constructs

Namalwa cells were transfected with blr1-Luc or blr1MH-Luc and incubated overnight, after which the cells were divided into three groups and reporter activity measured as indicated, either immediately (time point 0) or after incubation with or without okadaic acid for 7 h. The reporter activity at time point 0 was set to 1 in all experiments and the activity in cells incubated further is shown relative to this value. The bars represent the means $\pm$ S.D. of three independent experiments.

equal parts after overnight incubation. One part was used to measure luciferase activity after overnight incubation, whereas the remaining two parts were further incubated for 7 hours, either in the presence of 100 ng/ml okadaic acid or in its absence. Subsequently, the cells were lysed and the reporter activity was measured. On average, the reporter activity in the cells incubated for an additional 7 h was increased by over 50 % compared with that after the overnight incubation (Figure 5). However, no increase of luciferase expression was observed in cells incubated in the presence of okadaic acid. To test if this effect was due to the reduced binding of cellular Oct-2 protein to the recognition sequence in the promoter, we analysed in parallel the activity of the mutant reporter construct blr1MH-Luc. This construct differs from the blr1-Luc in a single nucleotide substitution, which was introduced into the Oct-2 binding site, changing its sequence to that of the human *BLR1* promoter. In contrast with the situation described above, addition of okadaic acid to the incubation medium did not completely abort the expression of luciferase from this mutant construct, although the reporter activity in the transfected cells incubated in the presence of okadaic acid was reduced compared with that in the cells incubated for the same time without okadaic acid (Figure 5).

Localization of the phosphorylated residues in Oct-2 protein

Localization of the sites of phosphorylation could provide a hint regarding the candidate kinases involved in phosphorylation of Oct-2, because many kinases phosphorylate target proteins on serine or threonine residues located within specific amino acid sequences. In an attempt to identify the amino acid residues that become phosphorylated in Oct-2, a GST-Oct-2 fusion protein was expressed in *E. coli* and phosphorylated *in vitro* with [γ - 32 P]-labelled dATP using crude lysates of Raji cells pretreated in culture for 2 h with 100 ng/ml okadaic acid. After electrophoretic separation in a SDS/polyacrylamide (10 %) gel the proteins which incorporated radioactively labelled phosphates were detected by autoradiography (Figure 6A). Two major phosphorylated forms of the GST-Oct-2 fusion protein were detected in the gel. The molecular mass of the faster-migrating form

Figure 6 Localization of the sites of phosphorylation in the GST-Oct-2 fusion protein

(A) The GST-Oct-2 fusion protein or the GST domain alone was expressed in *E. coli* and the proteins retained in the glutathione-agarose column were separated on a SDS/polyacrylamide gel and analysed by Coomassie Brilliant Blue staining (lanes 1 and 2). The proteins were phosphorylated *in vitro* with crude lysates of Raji cells in the presence of [γ - 32 P]dATP and autoradiographically detected upon separation by SDS/polyacrylamide gel (lanes 3 and 4); ns, non-specific. (B) Amino acid sequence of the Oct-2 part of the fusion protein shown in single-letter code. The POU domain is outlined with the grey shaded area. The arrows show the chymotryptic (Ch) and tryptic (T) peptides that were most heavily labelled by phosphorylation *in vitro* of the fusion protein and identified by Edman degradation. The sizes of peptides obtained by tryptic degradation were confirmed by MS. No mass data could be obtained from chymotryptic fractions. The potentially phosphorylated amino acid residues are shown with boxes. The black triangles indicate positions of Ser¹⁹⁷ and Thr²⁰⁸ residues.

appeared to correspond to that of the fusion protein prior to incubation with Raji cell lysates. The retarded form consisted of at least two separate bands that could be visualized by Coomassie Brilliant Blue staining of the gel. Both these bands produced a stronger radioactive signal than the faster-migrating form. These data suggest that phosphorylation of the GST-Oct-2 fusion protein causes a retardation of migration in SDS/polyacrylamide gels. Therefore phosphorylation of the recombinant fusion protein expressed in bacteria produces a similar effect to that observed with the native Oct-2 protein (compare to Figure 1A, lanes 1 and 2).

As a result of the proteolytic analysis of GST-Oct-2 fusion protein, five phosphorylated locations were identified within Oct-2 (Figure 6B). These include one peptide corresponding to the N-terminal part of the protein and four regions within the DNA-binding POU domain. Comparison of the identified phosphorylated peptide sequences with the database of the known preferred phosphorylation sites of different kinases [26] revealed two sites that match with the known patterns of phosphorylation. Both identified amino acid sequences lie within the POU-specific subdomain of Oct-2. They include the sequence Ser¹⁹⁷-Asp-Leu-Glu, which represents a preferred site of phosphorylation by casein kinase II (CK II), and the sequence Thr²⁰⁸-Phe-Lys, which is a potential site of phosphorylation by PKA.

DISCUSSION

Several lines of evidence suggest that phosphorylation of octamer factors may provide a mechanism of differential regulation of octamer factor binding to high- and low-affinity binding sites [14,16]. Here we analysed the effect of hyperphosphorylation of Oct-2 induced by okadaic acid on binding of this factor to three octamer sequences. One is a classical high-affinity binding octamer site present in the promoters of immunoglobulin genes or in the H2B gene promoter [6]; the other two are the non-canonical sequences identified in the promoters of the human and murine *BLR1* genes [20]. The latter two differ from each other by a single nucleotide substitution at position 3 and both have a substitution at position 2 compared with the classical motif 5'-ATGCAAT-3'. We showed that Oct-2 binds to the octamer site of the human *BLR1* promoter with higher affinity than to its murine counterpart and with much lower affinity to both these sites compared with the classical octamer motif. The obtained data reveal that phosphorylation of Oct-2 causes a dramatic reduction of its ability to bind to the murine *BLR1* non-canonical octamer sequence. At the same time, binding to the octamer sequence of the human *BLR1* promoter or to the classical octamer site remained unaffected under the same conditions. The latter observation contradicts the previously described data showing inhibition of Oct-2 binding to the octamer site of the H2B promoter [16]. However, the opposite effects of the okadaic acid treatment of intact cells and inhibition of phosphatases in nuclear extracts may reflect the differential activation of kinases *in vivo* and *in vitro*.

The exact nature of the kinases involved in the process of Oct-2 phosphorylation could not be revealed in this study. Search for the sites of phosphorylation in Oct-2 protein identified four candidate locations within the POU domain, which mediates contact to the major groove of DNA and plays a critical role in the high-affinity sequence-specific binding [8]. Two of the four identified phosphorylated regions lie within the POU-specific subdomain directly contacting the 5'-ATGC subsite of the octamer motif [27]. We showed that the interaction of phosphorylated Oct-2 with DNA is sensitive to nucleotide substitutions within this subsite. The identified amino acid stretches within the POU-specific subdomain contain preferred sequences of phosphorylation by CK II and PKA. However, the direct involvement of these kinases in the process of Oct-2 phosphorylation was not proved in the present study. The effect of Oct-2 hyperphosphorylation upon treatment of cells with okadaic acid could not be prevented by a specific inhibitor of PKA. Additionally, the Ser¹⁹⁷ → Ala or the Thr²⁰⁸ → Ala mutations of the CK II and PKA phosphorylation sites respectively were not able to abrogate inhibition of Oct-2 DNA binding caused by phosphorylation of this factor (results not shown). This suggests that the observed selective down-regulation of Oct-2 binding

may be the result of the synergistic effect of phosphorylation of several amino acid residues.

A possible explanation for the observed effect of Oct-2 phosphorylation on binding-site recognition may be a direct electrostatic interference of the phosphates coupled to the protein domain responsible for DNA contact, therefore preventing effective binding. However, our results indicate that the inhibitory effect of Oct-2 phosphorylation specifically depends on the presence of a nucleotide other than G in position 3 of the octamer sequence. This suggests a probable alteration of Oct-2 factor conformation upon phosphorylation, which renders this factor more restrictive to the nucleotide sequence of the recognition site compared with the unphosphorylated form of the protein. This scenario would resemble the effect of phosphorylation on the Pit-1 factor. Similar to Oct-2, phosphorylated Pit-1 exhibits a decreased binding affinity to selected binding sites and increased or unaltered binding to other recognition sites [14]. It was shown that the differential binding pattern of this factor is determined by a conformational change which leads to increased selectivity of the binding-site recognition dependent on the DNA sequences adjacent to the core motif.

The POU-specific domain is also responsible for protein-protein interactions with co-activators of octamer factors. It was demonstrated that the B-cell-specific co-activator of octamer-binding factors termed Bob1 (OBF-1/OCA-B) [28–32] potentiates Oct-2-mediated activation of the BLR1 promoter and is essential for the expression of this gene [20]. Bob1 selectively interacts with the POU-specific domain and the POU-homeo-domain of either Oct-1 or Oct-2, forming a ternary complex on the promoter DNA [33,34]. In addition Bob1 is able to contact the major groove of DNA on its own although this interaction is stabilized only in the presence of a POU domain [35]. Association of Bob1 with the octamer factors increases the selectivity of the nucleotide-sequence recognition compared with the free octamer factors [33].

One of the functions provided by the interaction of Bob1 with the POU domain is the stabilization of the POU-domain contacts on the DNA [34,36]. The structural requirements for selective interaction between octamer factors and their co-activator Bob1 and formation of the ternary complex have been investigated recently [34]. Intriguingly, the residues in the POU domain that are essential for establishing contact with Bob1 were mapped to the very beginning of the first helix of the POU-specific domain, a region where two identified phosphorylation sites are located. Furthermore, it was shown that the presence of a guanine residue at position 3 of the octamer sequence is critical for the formation of the ternary complex with Bob1 [34]. As described above, the same nucleotide position determines the effect of Oct-2 phosphorylation on its binding ability. This raises the interesting possibility that phosphorylation of the POU-specific subdomain of Oct-2 may conditionally interfere with the interaction with the Bob1 co-activator. This would still enable sufficient contact between both proteins and DNA when the octamer sequence possesses a guanine residue at position 3. However, it would prevent interaction of proteins when this position is occupied by another nucleotide. As a result the contact of Oct-2 with the octamer site would no longer be stabilized by Bob1 and consequently the binding of this factor to DNA would be down-regulated.

The expression of BLR1 on cultivated murine resting B cells or cell lines could be completely down-regulated upon stimulation by interleukin-6 or lipopolysaccharide [37]. Our data show that

down-regulation of Oct-2 binding to the promoter of the murine *BLR1* gene induced by hyperphosphorylation of this factor correlates with the dramatic reduction of the BLR1 mRNA level in murine splenic cells treated with okadaic acid. Therefore, phosphorylation of Oct-2 caused by activation of cellular kinases or inhibition of phosphatase activities may be one of the physiological mechanisms that mediates down-regulation of *BLR1* gene expression in mice upon activation of B cells.

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REFERENCES

- Bohmann, D., Keller, W., Dale, T., Schöler, H. R., Tebb, G. and Mattaj, J. W. (1987) *Nature* (London) **325**, 268–272
- Fletcher, G., Heintz, N. and Roeder, R. G. (1987) *Cell* **51**, 773–781
- Staudt, L. M., Singh, H., Sen, R., Wirth, T., Sharp, P. A. and Baltimore, D. (1986) *Nature* (London) **323**, 640–643
- Scheidereit, C., Heguy, A. and Roeder, R. G. (1987) *Cell* **51**, 783–793
- Müller, M. M., Ruppert, S., Schaffner, W. and Matthias, P. (1988) *Nature* (London) **336**, 544–551
- Herr, W. and Cleary, M. A. (1995) *Genes Dev.* **9**, 1679–1693
- Ruvkun, G. and Finney, M. (1991) *Cell* **64**, 475–478
- Verrijzer, C. P. and Van der Vliet, P. C. (1993) *Biochim. Biophys. Acta* **1173**, 1–21
- Hill, C. S. and Treisman, R. (1995) *Cell* **80**, 199–211
- Jackson, S. P. (1992) *Trends Cell. Biol.* **2**, 104–108
- Roberts, S. B., Segil, N. and Heintz, H. (1991) *Science* **253**, 1022–1026
- Segil, N., Roberts, S. B. and Heintz, N. (1991) *Cold Spring Harbor Symp. Quant. Biol.* **56**, 285–292
- Segil, N., Roberts, S. B. and Heintz, N. (1991) *Science* **254**, 1814–1816
- Kapiloff, M. S., Farkash, Y., Wegner, M. and Rosenfeld, M. G. (1991) *Science* **253**, 786–789
- Tanaka, M. and Herr, W. (1990) *Cell* **60**, 375–386
- Grenfell, S. J., Latchman, D. S. and Thomas, N. S. B. (1996) *Biochem. J.* **315**, 889–893
- Dobner, T., Wolf, I., Emrich, T. and Lipp, M. (1992) *Eur. J. Immunol.* **22**, 2795–2799
- Förster, R., Mattis, A. E., Kremmer, E., Wolf, E., Brem, G. and Lipp, M. (1996) *Cell* **87**, 1037–1047
- Förster, R., Emrich, T., Kremmer, E. and Lipp, M. (1994) *Blood* **84**, 830–840
- Wolf, I., Pevzner, V., Kaiser, E., Bernhardt, G., Claudio, E., Siebenlist, U., Förster, R. and Lipp, M. (1998) *J. Biol. Chem.* **273**, 28831–28836
- Pevzner, V., Wolf, I., Burgstahler, R., Förster, R. and Lipp, M. (1999) *Curr. Top. Microbiol. Immunol.* **246**, 79–85
- Schreiber, E., Matthias, P., Müller, M. M. and Schaffner, W. (1989) *Nucleic Acids Res.* **17**, 6419
- Laemmli, U. K. (1970) *Nature* (London) **227**, 680–685
- Solecki, D., Schwartz, S., Wimmer, E., Lipp, M. and Bernhardt, G. (1997) *J. Biol. Chem.* **272**, 5579–5586
- MacKintosh, C. and MacKintosh, R. W. (1994) *Trends Biochem. Sci.* **19**, 444–448
- Blom, N., Kreegipuu, A. and Brunak, S. (1998) *Nucleic Acids Res.* **26**, 384–386
- Klemm, J. D., Rould, M. A., Aurora, R., Herr, W. and Pabo, C. O. (1994) *Cell* **77**, 21–32
- Gstaiger, M., Knoepfel, L., Georgiev, O., Schaffner, W. and Hovens, C. M. (1995) *Nature* (London) **373**, 360–362
- Luo, Y. and Roeder, R. G. (1995) *Mol. Cell. Biol.* **15**, 4115–4124
- Strubin, M., Newell, J. W. and Matthias, P. (1995) *Cell* **80**, 497–506
- Pfisterer, P., Zwilling, S., Hess, J. and Wirth, T. (1995) *J. Biol. Chem.* **270**, 29870–29880
- Schubart, D. B., Sauter, P., Massa, S., Friedl, E. M., Schwarzenbach, H. and Matthias, P. (1996) *Nucleic Acids Res.* **24**, 1913–1920
- Gstaiger, M., Georgiev, O., van Leeuwen, H., van der Vliet, P. and Schaffner, W. (1996) *EMBO J.* **15**, 2781–2790
- Sauter, P. and Matthias, P. (1998) *Mol. Cell. Biol.* **18**, 7397–7409
- Cepek, K. L., Chasman, D. I. and Sharp, P. A. (1996) *Genes Dev.* **10**, 2079–2088
- Babb, R., Cleary, M. A. and Herr, W. (1997) *Mol. Cell. Biol.* **17**, 7295–7305
- Kaiser, E., Förster, R., Wolf, I., Ebensperger, C., Kuehl, W. M. and Lipp, M. (1993) *Eur. J. Immunol.* **23**, 2532–2539