Requirement for O-linked \(N\)-acetylglucosaminytransferase in lymphocytes activation

Alexander Golks, Thi-Thanh Thao Tran, Jean Francois Goetschy and Danilo Guerini*

Autoimmunity and Transplantation, Novartis Pharma AG, Basel, Switzerland

The dynamic modification of nuclear and cytoplasmic proteins with O-linked \(\beta\)-N-acetylglucosamine (O-GlcNAc) by the O-linked \(\beta\)-N-acetylglucosaminyltransferase (OGT) is a regulatory post-translational modification that is responsive to various stimuli. Here, we demonstrate that OGT is a central factor for T- and B-lymphocytes activation. SirNA-mediated knockdown of OGT in T cells leads to an impaired activation of the transcription factors NFAT and NFkB. This results in a reduction of IL-2 production consistent with prevention of T-cell activation. OGT is also required for the early activation of B cells mediated by stimulation of the B-cell receptor. Mechanistically, we demonstrate that NFkB as well as NFAT are glycosylated with O-GlcNAc after direct binding to OGT. Moreover, kinetic experiments show that O-GlcNAc modification prominently increased shortly after activation of lymphoid cells and it might be required for nuclear translocation of the transcription factors NFkB and NFAT.

The EMBO Journal (2007) 26, 4368–4379. doi:10.1038/sj.emboj.7601845; Published online 20 September 2007
Subject Categories: signal transduction; immunology
Keywords: lymphocytes activation; OGT; O-GlcNAc

Introduction

O-linked \(N\)-acetylglucosaminytransferase (OGT) is a glycosyltransferase that catalyzes the addition of a single \(\beta\)-N-acetylglucosamine (GlcNAc) to Ser/Thr residues of various nuclear and cytoplasmic proteins (Holt et al., 1987; Kreppel et al., 1997; Lazarus et al., 2006). The proteins targeted by O-linked \(\beta\)-N-acetylglucosamine (O-GlcNAc)-modification can be divided into groups according to their function. The main group includes proteins involved in transcription and translation (Love and Hanover, 2005). Accumulating evidence suggests that the O-GlcNAc modification of proteins plays a key role in regulating protein activity. In some cases, the site of O-GlcNAc and O-phosphate (Ser/Thr phosphorylation) was mapped to the same residues leading to the hypothesis of reciprocal regulation and competition of both modifications (Kelly et al., 1993; Chou et al., 1995; Medina et al., 1998; Cheng et al., 2000; Cheng and Hart, 2001; Federici et al., 2002). An enzyme with antagonistic function to OGT, cytosolic \(\beta\)-N-acetylglucosaminidase (O-GlcNAse), has been identified and it is ubiquitously expressed in all tissues (Gao et al., 2001).

Different splice variants of OGT are known, but their biochemical properties are not well characterized (Shafi et al., 2000). Knockout mice for OGT are embryonically lethal (Shafi et al., 2000), suggesting a critical function during mice development. Conditional knockout of OGT in T cells resulted in an increase of apoptotic T cells (O’Donnell et al., 2004). One study shows that the activation of lymphocytes with ConA or PMA/ionomycin leads to a rapid increase in nuclear and cytoplasmic glycoproteins (Kearse and Hart, 1991). Still only limited information is available on the role of O-GlcNAc modifications in immune cells.

In T cells, specific systems downstream of the T-cell receptor (TCR) complex lead to the activation of transcription factors that regulate a variety of activation-associated genes. Among these genes are cytokines and surface receptors that play an important role in coordinating the immune response (Crabtree and Clipstone, 1994). A proximal consequence of the engagement of TCR is the activation of PLC\(\gamma\), which catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphate in inositol 1,4,5-triphosphate and diacylglycerol. Whereas the latter mediates activation of the PKC family members and the Ras-MAPK pathway, inositol 1,4,5-triphosphate promotes an increase of intracellular Ca\(^{2+}\) concentration (Dower et al., 2000; Baier, 2003). The increase of Ca\(^{2+}\) activates calcineurin, a Ca\(^{2+}\)/calmodulin-dependent protein phosphatase, which dephosphorylates the transcription factor NFAT, allowing its nuclear translocation (Dower et al., 2000). In activated T cells, NFAT is a critical factor for transcriptional induction of IL-2 as well as other genes, for example, the cytokines IL-4, IFN\(\gamma\) and TNF\(\alpha\) (Wang et al., 1996; Kiani et al., 2001; Monticelli and Rao, 2002). The NFAT family of transcription factors includes NFAT1, NFAT2, NFAT3 and NFAT4. NFAT1 and NFAT2 are preferentially expressed in peripheral T cells (Lyakh et al., 1997; Masuda et al., 1998).

The transcription factor NFkB regulates the expression of genes crucial for innate and adaptive immune responses, cell growth and apoptosis (Ghosh and Karin, 2002). The NFkB dimer is retained in the cytosol by interaction with the inhibitor of \(\kappa\)B protein (I\(\kappa\)B) complex. Its nuclear translocation can be induced by a wide variety of stimuli, for example, TCR or B-cell receptor (BCR) activation (Hayden and Ghosh, 2004). TCR or BCR ligation leads to activation of the I\(\kappa\)B kinase (IKK) complex, which consists of two catalytic subunits, I\(\kappa\)K\(\alpha\) and I\(\kappa\)K\(\beta\), as well as a regulatory subunit, I\(\kappa\)K\(y\)/NEMO. Upon I\(\kappa\)B phosphorylation, I\(\kappa\)Bs are degraded in a...
ubiquitin-dependent manner, allowing the nuclear translocation of NFκB (Hayden and Ghosh, 2004).

Here, we show that NFκB and NFAT are O-GlcNAc-modified by OGT by direct interaction. A large increase of O-GlcNAc-modified NFAT was observed shortly after T-/B-cell activation, which correlated well with its translocation to the nucleus. Moreover, silencing of OGT results in impaired activation of T and B lymphocytes. Overexpression of OGT as well as the use of inhibitors of the antagonistic enzyme O-GlcNAcase sensitized T and B cells toward activation.

Results

**SiRNA-mediated downregulation of OGT results in impaired IL-2 production and reduced CD69 surface expression in T cells**

To analyze the role of OGT on TCR signaling, we took advantage of the siRNA technology to downregulate lymphocyte protein tyrosine kinase (Lck) as positive control or OGT. The efficiency of siRNA-mediated downregulation of OGT and Lck was analyzed using real-time PCR (Figure 1A). The
amount of mRNA after treatment with siRNA (in percent) was compared with the amount of mRNA in cells transfected with a control siRNA (Alexa Fluor 488, QiAGEN) set as 100%. In parallel, the effect of the siRNA on the endogenous IL-2 transcription was measured as an indication of T-cell activation. OGT siRNA (OGT_1) was used in these experiments, but similar results were observed with OGT_2 and _3, control siRNA or siRNA specific for Lck were transfected into Jurkat cells. The cells were stimulated 24, 48 or 72 h after transfection with plate-bound anti-CD3/28 antibodies and mRNA was prepared. Real-time PCR experiments revealed efficient OGT downregulation, which was paralleled by markedly reduced IL-2 transcription (Figure 1A, upper panel); IL-2 mRNA was reduced up to 60–70%. Lck siRNA also showed efficient downregulation of the corresponding transcript and the mRNA for IL-2 (Figure 1A, lower panel). In summary, these data indicate that OGT affects TCR signaling, and downregulation of OGT results in impaired transcription of IL-2.

To visualize the siRNA-mediated downregulation of OGT at protein level, we immunoprecipitated OGT and analyzed the precipitates by Western blotting. Jurkat cells were transfected with siRNA specific for OGT and cultured for 24–72 h prior lysis and immunoprecipitation. Downregulation was detected already after 24 h of incubation and to a higher extent after 48 and 72 h (Figure 1B).

For further analysis of OGT’s influence on T-cell activation, we took advantage of a luciferase reporter system that was under the control of the IL-2 promoter. Jurkat cells expressing the IL-2 reporter plasmid were transfected with either a control siRNA or siRNAs specific for Lck or OGT. The cells were incubated for 24 h to ensure downregulation of OGT by the siRNA. Transfection with a control siRNA did not affect TCR stimulation induced IL-2 promoter activity (Figure 1C). Lck silencing was again used as a positive control, since the well-established Lck plays an essential function in TCR signaling (Bijlmakers and Marsh, 2000). Silencing of Lck results in 40% decrease in activity of the IL-2 promoter. Similar results (up to 40% decrease of IL-2 promoter activity) were obtained for three independent siRNAs against OGT (Figure 1C), suggesting an important role of OGT in T-cell activation.

We further investigated the cell-surface expression of CD69, a marker of early T-cell activation. As visualized in Figure 1D, the downregulation of OGT (three active siRNA for OGT were used) results in a reduced surface expression of CD69 upon TCR stimulation. CD69 surface expression upon stimulation with PMA/ionomycin (bypassing TCR signaling) was neither depending on OGT nor Lck (Figure 1E). In summary, our data suggest OGT as a critical factor involved in T-cell activation.

**Modulation of OGT affects early activation of B and T lymphocytes**

We examined if OGT also plays a critical role in the activation process of B lymphocytes. As for T cells, CD69 surface expression is a hallmark of early B-cell activation. Jurkat and BJAB cells were transfected with either a control siRNA or siRNA against OGT and cells were rested for 24 h. After TCR stimulation, Jurkat cells treated with OGT siRNA showed reduced surface expression of CD69 after 8 and 16 h of stimulation (Figure 2A). At 24 h after transfection, BJAB cells were stimulated with anti- IgM antibodies and like in T cells, a reduced surface expression of CD69 was detected (Figure 2B).

In parallel, we evaluated the effect of overexpression of OGT. Cells were transfected using the Amaxa method with a plasmid containing the full-length OGT or GFP as a control, cultured for 48 h and then lysed. OGT overexpression was confirmed by Western blotting (Figure 2C). The lysates were also blotted for O-GlcNAc levels which are not increased upon overexpression of OGT, most likely because the levels of OGT’s substrate UDP-N-acetylglucosamine remain unchanged.

We next examined whether overexpression of OGT might sensitize BJAB cells toward activation. As a readout for activation, we used a luciferase NFxB reporter assay. A plasmid encoding a luciferase gene under the control of NFxB promoter was cotransfected with OGT or GFP. The overexpression of OGT leads to an enhanced BCR-dependent activation of NFxB (Figure 2D). We also checked for increased CD69 surface externalization. Cells were transfected as described before and cultured for 48 h. After 48 h, cells were stimulated with anti-IgM antibodies. Overexpression of OGT leads to a higher CD69 surface externalization than control-transfected cells (Figure 2E).

Whereas the downregulation of OGT in lymphocytes leads to an impaired activation, the overexpression rather sensitizes lymphocytes toward activation. To analyze whether the effects of OGT are specific for lymphocytes, similar luciferase reporter gene assays as described in Figure 2D were performed in HEK293, COS-1 and CHO cells. In none of these three cell lines, the expression of OGT showed a significant influence on NFxB activity (data not shown).

**The TCR-induced activation of the transcription factors NFAT and NFkB is impaired upon OGT downregulation**

Among others, IL-2 mRNA transcription is dependent on the two transcription factors, NFAT and NFkB (Torgerson et al., 1998). To evaluate if the effect of OGT on IL-2 was dependent on NFAT and/or NFkB, Jurkat cells stably expressing a luciferase reporter plasmid for NFAT or NFkB were used. The cells were transfected with either a control siRNA or siRNAs for Lck or OGT. The cells were further cultured for 24, 48 or 72 h, respectively, to ensure optimal downregulation of Lck and OGT. After TCR stimulation of the cells, the activation of NFAT (Figure 3A) and NFkB (Figure 3B) was quantified by luciferase assay. The activity of both transcription factors upon TCR triggering was reduced by siRNA specific for Lck. Three independent siRNAs against OGT also led to impaired activation of NFAT (Figure 3A) and NFkB (Figure 3B).

**NFkB and NFAT are O-GlcNAc-modified**

As OGT seems to be required for the transcriptional activity of NFkB and NFAT, we probed for O-GlcNAc modifications of NFkB and NFAT. NFkB was described to be O-GlcNAc-modified in rat mesangial cells (James et al., 2002). To confirm this observation in human T cells, we immunoprecipitated NFkB with an antibody specific for the p65 chain and probed for the presence of the O-GlcNAc residue (Figure 4A). As a control immunoprecipitation, we used an antibody to c-myc as irrelevant antibody. O-GlcNAc modification of NFATc1 was analyzed in a similar manner. NFATc1 was immunoprecipitated and analyzed for O-GlcNAc modifications. These
experiments show that also NFATc1 is specifically detected by an antibody raised against O-GlcNAc (Figure 4B). Immunoprecipitations with two antibodies to O-GlcNAc revealed the presence of modified proteins in lymphocyte lysates, among which NFkB-p65 and NFATc1 were identified (Figure 4C). The specificity of the anti-O-GlcNAc antibody used for detection of O-GlcNAc-modified NFkB and NFATc1 was verified in Western blots. Preincubation of the anti-O-GlcNAc antibody RL-2 with an excess of N-acetylglucosamine, completely competed the signal for O-GlcNAc-modified NFkB-p65 (Figure 4D) and NFATc1 (Figure 4E).

**OGT interacts with NFkB and NFATc1**

We were further interested in whether OGT might interact with NFkB and NFATc1. Immunoprecipitations of NFkB and NFATc1 were blotted for the presence of OGT, using the two

---

**Figure 2** OGT knockdown impairs, whereas overexpression sensitizes T-cell and B-cell toward early activation. (A) Jurkat cells were transfected either with a negative control siRNA (Ctrl) or siRNA specific for OGT (OGT_1). After transfection, the cells were transferred to fresh culture medium for 24 h before stimulation with plate-bound anti-CD3/28 antibodies for 8 or 16 h, respectively. After stimulation, CD69 surface expression was measured by Flow cytometry as described in Materials and methods. Experiments were performed in triplicates. CD69 externalization after OGT or Ctrl transfection is given as a percentage of the maximal externalization obtained by treating with PMA/ionomycin, set as 100%. (B) BJAB cells were either transfected with a negative Ctrl or siRNA specific for OGT (OGT_1). After transfection, the cells were transferred to fresh culture medium for 24 h before stimulation with anti-IgM antibodies for 8 or 16 h, respectively. After stimulation, CD69 surface expression was measured as in (A). (C) BJAB cells were transfected with plasmids encoding GFP as a control or OGT and transferred to fresh medium for 48 h. With an antibody to the V5 tag, total cellular lysates were analyzed for expression of OGT. NFkB-p65 was blotted as loading control. The overall amount of O-GlcNAc levels were blotted with antibodies to O-GlcNAc (RL-2). (D) BJAB cells were cotransfected with a luciferase reporter plasmid encoding a NFkB reporter together with either GFP (as a control) or full-length OGT. Cells were incubated for 24 h and stimulated with anti-IgM antibodies for additional 12 h (+) or left unstimulated (−). After lysis, the luciferase activity was measured as described in Materials and methods. Experiments were performed in triplicates. The maximal NFkB activity observed after OGT overexpression and anti-IgM stimulation was set to 100%. (E) BJAB cells were transfected with plasmids for the overexpression of GFP (as a control) or of the full-length OGT. Cells were transferred to fresh medium for 48 h and stimulated with anti-IgM antibodies for 12 h. The maximal CD69 externalization observed after OGT overexpression and anti-IgM stimulation was set to 100%.
antibodies TI-14 and DM-17. This experiment showed interaction of OGT to NFκB and NFATc1 (Figure 5A). Vice versa, OGT-IPs using two different antibodies were performed and co-precipitated NFκB and NFAT were detected in a Western blot, confirming the binding of OGT to both transcription factors (Figure 5B).

In summary, these data show that in lymphocytes NFκB as well as NFAT are O-GlcNAc-modified and the glycosylation is likely to occur after binding of OGT.

**NFATc1 is O-GlcNAc-modified by OGT in vitro**

To confirm that NFATc1 is O-GlcNAc-modified by OGT, we established an in vitro glycosylation assay. OGT was over-expressed in baculovirus-infected S9/H5 cells and lysates enriched in OGT were used for the glycosylation assay. A lysate of uninfected cells was used as a control. A C-terminal fragment of the rat Nup62 expressed and purified in bacteria was used as a substrate for OGT (Marshall et al., 2003). The composition of the whole reaction mix was separated by SDS–PAGE and it is shown in Figure 6A after Coomassie brilliant blue staining (upper panel). Radioactive UDP-[3H]GlcNAc was added to the reaction mix, incubated for 30 min at 37°C, and the modification of Nup62 with [3H]GlcNAc was visualized after SDS–PAGE and autoradiography (Figure 6A, lower panel). The modification of Nup62 is only seen in the presence of OGT and Nup62 (left lane). No modification (radioactive band) was detected in either an assay performed in the absence of Nup62 (middle lane) or in the absence of OGT (right lane). A similar assay was performed using recombinant NFATc1 as a substrate for OGT (Figure 6B). The composition of the reaction mix was visualized after SDS–PAGE and a Coomassie brilliant blue staining (Figure 6B, upper panel). O-GlcNAc-modified recombinant NFATc1 was detected in the autorography (lower panel, first lane). In the absence of either OGT (lane 2) or recombinant NFATc1 (lane 3), no radioactive band with the size of NFATc1 was detected.

In parallel, the same reaction mixture was precipitated with TCA and counted in a liquid scintillation counter. The increase in radioactivity paralleled increasing concentrations of the substrate Nup62 (Figure 6C). The same assay was performed with 0–2 μg of recombinant NFATc1 as a substrate. The results strongly suggest that recombinant NFATc1 was O-GlcNAc-modified by OGT (Figure 6D).
OGT is a key mediator of lymphocytes activation

NFATc1 is transiently modified shortly after lymphocytes stimulation

Next, we decided to analyze the kinetics of O-GlcNAc-modification of NFATc1 upon TCR stimulation. Jurkat cells were costimulated via the TCR and aliquots were retrieved at regular intervals. Immunoprecipitation of NFATc1 was performed and probed with an antibody recognizing O-GlcNAc-modified proteins (Figure 7A). As early as ~5 min after

**Figure 4** NFATc1 and NFkB are O-GlcNAc-modified. (A) Western blot analysis of O-GlcNAc modifications of NFkB-p65 after immunoprecipitation (5 × 10^7 Jurkat cells each) using anti-NFkB antibodies against p65 subunits of NFkB and irrelevant antibodies (anti-c-myc) as a negative control. To visualize the background bands of the antibodies used for IP, we did IPs omitting the lysate. Positions of NFkB subunits as that of IgGH are indicated. (B) Western blot analysis of O-GlcNAc modifications after immunoprecipitation (5 × 10^7 Jurkat cells each) using an anti-NFATc1 antibody or irrelevant antibodies (anti-c-myc) as a negative control. To visualize the background bands of the antibodies used for IP, we did IPs omitting the lysate. Positions of NFATc1 proteins are indicated. (C) Western blot analysis of NFkB-p65, NFATc1 and O-GlcNAc modifications after immunoprecipitation (5 × 10^7 Jurkat cells each) using anti-O-GlcNAc antibodies (RL-2 and HGAC85). Positions of NFkB-p65, NFATc1 and IgGH are indicated at the right side of the panels. To visualize the background bands of the antibodies used for IP, we did IPs omitting the lysate. Antibodies used in the immunoprecipitation reactions are given at the top, whereas the antibodies used in the Western blots are at the left side. (D) Western blot analysis of O-GlcNAc modifications after immunoprecipitation (5 × 10^7 BJAB cells each) using an anti-NFATc1 antibody. The Western blot was developed with or without preincubation of the anti-O-GlcNAc antibody in 500 mM GlcNAc. (E) Western blot analysis of O-GlcNAc modifications after immunoprecipitation (5 × 10^7 BJAB cells each) using an anti-NFkB-p65 antibody. The Western blot was developed with or without preincubation of the anti-O-GlcNAc antibody in 500 mM GlcNAc.

**Figure 5** OGT binds to NFATc1 and NFkB. (A) Western blot analysis of OGT, NFATc1 and NFkB-p65 after immunoprecipitation (5 × 10^7 Jurkat cells each) using antibodies to NFkB and NFATc1. (B) Western blot analysis of OGT, NFATc1 and NFkB-p65 after immunoprecipitation (5 × 10^7 Jurkat cells each) using antibodies to OGT (DM-17 and TI-14).
OGT is a key mediator of lymphocytes activation

A Golk et al

Figure 6 NFATc1 is O-GlcNAc-modified by OGT in vitro. An O-
GlcNAc transferase assay was developed using a SF9/H5 lysate
containing enriched OGT expressed by baculovirus. Recombinant
Nup62 was used as a control substrate for OGT. (A) Upper panel:
Coomassie brilliant blue stained SDS-PAGE gel of whole reaction
mix (SF9/H5 lysates with or without OGT and Nup62). Lower panel:
the gel (the portion containing Nup62) is shown) was processed to
autoradiography after incubation with UDP-[3H]GlcNAc of the
lysate containing OGT or the gel (the portion containing Nup62
is shown). (B) Upper panel: Coomassie brilliant blue stained
SDS-PAGE gel of whole reaction mix (SF9/H5 lysates with or
without OGT and recombinant NFATc1). Lower panel: the gel
(the portion containing recombinant NFATc1 is shown) was processed to
autoradiography after incubation with UDP-[3H]GlcNAc of the
lysate containing OGT or lysate from SF9 cell infected with a control
virus (SF9 lysate). (C) The O-GlcNAc transferase assay was
performed as described in (A) with 0-10 μg of purified Nup62. The
reaction mixture was precipitated by TCA and the radioactivity
associated with the pellet counted. The amount of radioactivity
associated with the TCA precipitate correlates with the amount
of Nup62 present in the transferase reaction. (D) An assay similar
to that described in (B) was performed with 0, 1 and 2 μg of recombi-
nant NFATc1. The reaction mixture was precipitated by TCA and the
radioactivity associated with the pellet counted. The amount of radioactivity
associated with the TCA precipitate correlates with the amount
of NFATc1 present in the transferase reaction.

stimulation, an increase of O-GlcNAc-modified NFATc1 was
observed. The level of modified NFATc1 returned to base line
levels by 15-20 min after stimulation. As OGT is also in-
volved in B-cell activation, we evaluated time-dependent
changes in O-GlcNAc modification of NFATc1 upon BCR
stimulation. A large transient increase in the level of modified
NFATc1 was observed shortly after IgM stimulation of BJAB
cells (Figure 7B). Finally, the same type of experiment was
conducted in primary human T lymphocytes. As in Jurkat and
BJAB cells, a large transient increase in levels of modi-
ification of NFATc1 was observed after TCR stimulation
(Figure 7C). Quantitation of Western blots revealed that
after 5-10 min the amount of O-GlcNAc-modified NFATc1
increased ~14-fold in Jurkat T cells and ~4-fold in BJAB
cells or primary human T cells (Figure 7A-C, lowest panels).
The data show for the first time a large and transient OGT
modification of NFATc1 in lymphocytes after stimulation of
BCRs and TCRs.

We then investigated if kinetics of O-GlcNAc modification
might correlate with translocation of NFATc1 into the nucleus.
Cytosolic as well as nuclear extracts were prepared from
Jurkat cells stimulated for short periods of time via the
TCR. Immunoprecipitations of NFATc1 coupled to Western
blot analysis with an antibody to O-GlcNAc were performed
(Figure 7D). In the cytosol, levels of O-GlcNAc-modified
NFATc1 increased ~5 min after TCR stimulation. In the
nucleus, the bulk of O-GlcNAc-modified NFATc1 appeared
by 10-15 min, with a shift of 5-10 min compared to the
cytosol. The overall amounts of NFATc1 protein are reported
in the lower panel of Figure 7D. Nuclear NFATc1 total protein
concentration increased in parallel to the O-GlcNAc modifica-
tion, while changes in the cytosol were much less prominent.
Cytosolic and nuclear fractions were quantified by Western
blots using the anti-GAPDH antibodies for cytosolic fractions
and anti-Histone-H2B antibodies for nuclear fractions
(Figure 7D).

In summary, the data suggest that OGT modification upon
short-term stimulation of lymphocytes might be a prerequi-
site for nuclear translocation of NFATc1. In primary human
T cells, the lowest of the three NFATc1 bands (likely the
dephosphorylated form of NFATc1) seems to be preferentially
modified by O-GlcNAc. It can be speculated that dephos-
phorylated NFATc1 is the preferred substrate for modifica-
tion with O-GlcNAc.

The inhibitor of O-GlcNAcase, PUGNAc, sensitizes
toward early B-cell activation

OGlcNAc modifications are controlled by the transferase
OGT and by a protein with antagonistic function, O-
GlcNAcase, which removes this sugar residue from proteins
(Hanover, 2001). We used PUGNAc (O-(2-Azetimido-2-
deoxy-D-glucopyranosylidene)amino N-phenylcarbamate), a
specific inhibitor of O-GlcNAcase, predicting that it should
produce similar effects to those observed after overexpression
of OGT (Vosseller et al, 2002). PUGNAc treatment should
result in the increase of the level of O-GlcNAc modifications
of NFκB and NFAT and therefore facilitate the activation of
lymphocytes.

BJAB cells were treated with 100 μM PUGNAc for 0, 3, 6 or
12 h, respectively, and total cellular lysates were analyzed for
the level of O-GlcNAc-modified proteins (Figure 8A). Western
blot analysis of these lysates with antibodies against O-
GlcNAc demonstrated a time-dependent increase of
O-GlcNAc-modified proteins (Figure 8A). Upon treatment
with PUGNAc, the level of O-GlcNAc-modified NFκB was
also increased, whereas the overall amounts of immunopre-
Precipitated NFκB was not influenced by PUGNAc (Figure 8B, lower panel). Flow cytometry analysis of the cells treated with 100 μM PUGNAc for up to 72 h did not affect proliferation of the cells (data not shown). We then examined the consequence of the inhibition of O-GlcNAcase with PUGNAc on early B-cell activation, by evaluating CD69 surface expression. BJAB cells were either left untreated or were treated for 5 h with the inhibitor before stimulation with anti-IgM antibodies for 0, 12, 15 or 19 h, respectively. Treatment of BJAB cells with PUGNAc accelerated CD69 externalization, whereas treatment with PUGNAc alone did not result in an increase in CD69 surface externalization (Figure 8C). To confirm that inhibition of O-GlcNAcase facilitated NFAT activation, BJAB.NFAT.GFP cells were used. These cells express GFP under the control of a NFAT promoter allowing activation of the NFAT promoter to be analyzed by Flow cytometry analysis. BJAB.NFAT.GFP cells were either left untreated or treated with PUGNAc for 5 h and subsequently stimulated with anti-IgM antibodies for 12 h. Prominent GFP expression can be observed upon stimulation by anti-IgM antibodies (example in upper panel Figure 8D). In the presence of PUGNAc, a prominent increase of double-positive cells, GFP+ and CD69+, is observed (Figure 8D, lower panel). Thus, pretreatment with PUGNAc leads to an increase in NFAT activation as well as CD69 surface externalization.

Figure 7 NFATc1 is O-GlcNAc-modified before entry into the nucleus. (A) Jurkat T cells were stimulated with a mixture of anti-CD3/anti-CD28 antibodies and total cellular lysates were prepared. NFATc1 immunoprecipitations were performed from these lysates. After separation by SDS-PAGE the immunoprecipitates were analyzed by Western blot using antibodies to O-GlcNAc (RL-2) and NFATc1. Quantification of the Western blots is shown in the lowest panel. (B) BJAB cells were stimulated with anti-IgM antibodies and total cellular lysates were prepared. NFATc1 immunoprecipitations were performed from these lysates and analyzed as described in (A). (C) Primary human PBMCs were costimulated with a mixture of anti-CD3/anti-CD28 antibodies and analyzed as described in the legend to panel (A). (D) Jurkat cells were stimulated for short times with anti-CD3/28 antibodies. Cytosolic and nuclear extracts were prepared. The cytosolic as well the nuclear fractions were used for NFATc1 immunoprecipitations. The purity of the cytosolic and nuclear extracts was controlled by doing Western blot of GAPDH (cytosolic fractions) and Histone H2B (nuclear fractions).
The inhibitor of O-GlcNAcase, PUGNAc, sensitizes primary human lymphocytes toward activation

PUGNAc was used to test the effects of O-GlcNAc modification on the activation of primary human lymphocytes. Human PBMCs were treated with PUGNAc, T and B cells in PBMCs were identified by anti-CD3 and anti-CD19 antibodies, respectively, and then analyzed for CD69 surface expression. The inhibition of O-GlcNAcase by PUGNAc leads to a concentration-dependent increase in activation of primary human B cells (Figure 8E) and primary human T cells (Figure 8F). In summary, an increase of O-GlcNAc modifications upon inhibition of O-GlcNAcase leads to a sensitization of T as well as B cells toward TCR- and BCR-induced activation.

Discussion

This study demonstrates that the balance of O-GlcNAc modifications of the transcription factors NFAT and NFκB is decisive for lymphocytes activation. Proper levels of O-GlcNAc modifications are controlled by the O-GlcNAc transferase OGT and its counter-enzyme O-GlcNAcase. SiRNA-mediated downregulation of OGT leads to the impairment of T- and B-lymphocytes activation. Overexpression of OGT sensitizes lymphocytes toward activation similarly as the inhibition of O-GlcNAcase, the enzyme with antagonistic function to OGT. Mechanistic experiments showed that OGT interacts with NFAT as well as NFκB, thereby modifying both transcription factors. The effect of OGT on NFκB seems to be...
specific to lymphocytes, as we failed to reproduce a similar effect in other cells. This suggests that TCR/BCR-mediated signaling is required for the functional effect of OGT on NFAT/NFkB transcription factors. Moreover, a transient increase of O-GlcNAc modification of NFAT occurs upon TCR/BCR stimulation in the cytosol of lymphocytes, that seems to precede its translocation into the nucleus. In summary, our study for the first time points toward OGT as a key signaling component downstream of the TCR and the BCR.

O-GlcNAc modifications of proteins have been described for the first time around 20 years ago (Torres and Hart, 1984). The slow progress in this field can be mostly due to a lack of efficient methods for detection of O-GlcNAc-modified proteins. The currently fast growing number of O-GlcNAc-modified proteins includes enzymes, kinases, transcription factors and proteasome components (Zachara and Hart, 2006). Nevertheless, the role of O-GlcNAc modifications in lymphocytes signaling is poorly understood. Tissue-specific deletion of the OGT gene in mice resulted in a decrease of total number of CD4 T cells, which was thought to be mediated by increased apoptosis. The same study demonstrated that OGT does not affect the development of immature CD4/CD8 thymocytes (O’Donnell et al, 2004). The results with these transgenic mice are consistent with the function of OGT during T-cell activation described in this manuscript. The lack of OGT results in an incomplete or improper activation of peripheral T cells, increasing their chance to move toward apoptosis. The role of OGT is likely restricted to T-cell activation, as no defect was observed in immature thymocytes, which have not yet needed TCR-dependent signaling for positive selection.

OGT-catalyzed modifications of proteins are described to play prominent roles in many signaling pathways. The level of O-GlcNAc modifications has a direct link to glucose homeostasis, implicating a role of the OGT/O-GlcNAcase system in diabetes (Roos et al, 1997; Deaciu et al, 2004; Hanover et al, 2005). A putative role of O-GlcNAc in controlling the mitotic progression was also described (Slawson et al, 2005). Still limited information is available on the biochemical consequences of the O-GlcNAc modification of proteins, for example, the effects on the activity/function of target enzymes. Recently, it was reported that the modification with O-linked N-acetylgalcosamine regulates the activity and stability of the tumor suppressor p53 (Yang et al, 2006). The O-GlcNAc modification of S149 leads to a decreased phosphorylation at T155. Lowering phosphorylation at this site results in decreased p53 ubiquitination/proteolysis and, thus, higher stability of the protein (Yang et al, 2006). Another study suggests that O-GlcNAc modification of the transcription factor Elf-1 is regulated by O-GlcNAc modification together with phosphorylation and that both modifications are needed for nuclear translocation (Juang et al, 2002). In the case of NFAT, O-GlcNAc modification is also likely to play a role in its nuclear translocation. Dephosphorylation of Ser/Thr residues by calcineurin upon TCR activation unmasks signals that allow NFAT to translocate into the nucleus. As O-GlcNAc modifications have been shown to compete for sites recognized by kinases, O-GlcNAc might prevent rephosphorylation in the cytosol, therefore, favoring the translocation of a ‘primed’ state of NFAT. Or else, O-GlcNAc modifications promote a change in the conformation of NFAT that is prone to translocation. NFkB requires also phosphorylation/dephosphorylation events in order to be fully activated and to transduce into the nucleus. Upon phosphorylation, members of the IKK complex need to dissociate and to be degraded allowing NFkB to move to the nucleus. Enhanced dissociations might result from O-GlcNAc modification of NFkB. The detailed analysis of the underlying steps is a matter of future studies.

Interestingly, OGT is expressed at high levels in T cells and dendritic cells (data not shown). Despite being an essential gene for the survival of cells (Shafi et al, 2000), the high concentration of OGT suggests yet to be discovered additional and specific functions in T cells as well as dendritic cells. The novel role of OGT/O-GlcNAcase in lymphoid cells described above suggests that manipulation of this enzyme system might represent a novel way to therapeutic immunosuppression.

Materials and methods

Antibodies and reagents

The following antibodies were used: anti-NFkB p65 antibodies and anti-NFkB p50 antibodies (SC109 and SC-8414, Santa Cruz Biotechnology Inc); anti-NFATc1 antibodies (7A6, Alexis Biochemicals); anti-NFATc3 antibodies P4G5 and RL-2 (ab2735 and ab2739, Abcam); anti-OGT antibodies (DM-17 and TI-14, Sigma-Aldrich); anti-V5 antibodies (B960-25, Invitrogen); anti-CD60-APC; anti-CD3-FITC, anti-CD19-PECy5 antibodies (BD Biosciences); anti-Histone-H2B (IMG-359, Imgenex); anti-GAPDH (6C5, Ambion); anti-myc (9E10, Roche). PUGNAC was from Toronto Research Chemicals. Secondary Horseradish peroxidase-conjugated antibodies were from Cell Signaling. All other chemicals used were highest analytical grade available and from Merck or Sigma-Aldrich.

Cell lines

The Jurkat T cell line (clone E6.1), the BJAB B lymphoblastoid cell line and primary PBMcs (prepared by Ficoll density centrifugation using Leucosep, Greiner) were maintained in RPMI 1640, 10 mM sodium pyruvate, nonessential amino acids, 50 μg/ml gentamcin (all purchased from Gibco Life Technologies) and 10% fetal calf serum (PAA Technologies) in 5% CO₂, 37°C. Jurkat cells stably expressing the vector pGL2-neo-IL-2 as part of the IL-2 promoter (kindly provided by P Wenner, Novartis Pharma AG) as well as the BJAB.NFAT.GFP cell line expressing GFP under a NFAT promoter were maintained in culture medium with addition of 0.5 mg/ml G418 (Gibco Life Technologies). Jurkat cells stably expressing the NFkB- and NFAT-reporter plasmids (kindly provided by J-P Evenou, Novartis Pharma AG) were maintained in culture medium with addition of 0.25 mg/ml Hygromycin (Gibco Life Technologies). S9/ H5 cells were maintained in TNM-FH Insect Culture Medium with 10% FCS and 50 μg/ml gentamcin in a 27°C humidified incubator.

The IL-2-Luc Jurkat cell line was generated after stable transfection of Jurkat E6.1 with the vector pGL2-neo-IL-2. This vector was prepared by insertion of the IL-2 promoter region (−583 to +40 of human IL-2 promoter) in front of (HindIII site) to the lucerase reporter gene in the vector pGL2-Basic (Promega). The G418 resistance gene was transferred from the vectors pCMNeoPo- lyA (Stratagene) into the pSDF-β-gal site. pNFAT-Luc were purchased from Stratagene and used to prepare Jurkat stable cell lines. The NFAF-GFP construct used for the BJAB.GFP.NFAT cells was similar to the pNFAT-LUC available from Stratagene.

Protocols for activation of T and B cells

T cells were stimulated with plate-bound anti-CD3 and anti-CD28 antibodies, B cells with 10 μg/ml anti-IgM antibodies (Dr Andreas Katapodis, Novartis Pharma AG) for 5.5 h at 37°C. Alternatively, cells were stimulated with 10 ng/ml PMA with 2.5 μM ionomycin. For coating, flat bottom 96-well microtiter plates (Microlite 2+, Thermo Electron Corp.) were incubated with 3 μg/ml goat anti-mouse IgG Fc antibodies (Dianova) for 2–3 h, washed with PBS and incubated with 20 ng/ml anti-TCR/Ab antibodies (Becton Dickinson) and 600 ng/ml anti-CD28 antibodies (BD Biosciences) overnight at 4°C.
Western blot and immunoprecipitation

Western blot analysis as well as immunoprecipitations were performed as described by Laemmli (1970) and Golks et al. (2006). PVDF membranes were used according to the manufacturer’s protocol (Biorad). The antibodies were diluted as follows: anti-NFkB p65 and p50 antibodies (1:50); anti-NFATC1 antibodies (1:100); anti-GlcNAc antibodies H3AC8S or RL-2 (1:100); anti-GlcNAc antibodies DM-17 or TI-14 (3 μg/ml); anti-myc (2 μg/ml). In case of preincubation of the antibody to O-GlcNAc (RL-2) with N-acetylcysteine, 500 μM GlcNAc were dissolved in PBS-T and the antibodies preincubated for 30 min at room temperature. Nuclear and cytosolic extracts were prepared according to the manufacturer’s protocol using the Neon Ex Ten kit (ActiveMotif).

Quantification of Western blots was performed using the GeneTools Analysis Software version 3.02.00.

Flow cytometry analysis

CD69 surface staining was performed on 10^6 cells. The cells were washed with PBS, then incubated for 20 min in blocking solution (2% FCS/PBS), and stained with 1 μl anti-CD69-APC in 20 μl 2% FCS/PBS and analyzed using Flow cytometry. To evaluate the percentile of T cells and B cells, PBMCs were stained with anti-CD3-FITC or anti-CD19-PECy5 antibodies, respectively.

Cloning and expression of OGT in insect cells

OGT was amplified from the vector pQL-OGT-r8-C1_7-1 (Dr Brian Cloning and expression of OGT in insect cells using the following primer pairs: OGT_F: 5’-CTCTAGATATAGCCATTC; OGT_R_NO: 5’-CA TC. The PCR fragments were digested with Sall–EcoRI and EcoRI–EcoRV and ligated into pENTR2h. The full-length OGT was transferred to the pDEST10 vector by gateway reaction and the expression of OGT was performed according to the manufacturer’s protocol (Invitrogen).

Cell disruption and enrichment of OGT

OGT protein was isolated from 2–3 × 10^9 infected S9/H5 insect cells for 48 h. The cells were washed once in PBS, then lysed in 10 mM Tris–HCl, pH 7.5 (1 × 10^6 cells/ml). After addition of 1 mM DTT and protease inhibitors (Complete, Roche), cells were homogenized and diluted with one volume of 10 mM Tris–HCl, pH 7.5, 20% sucrose, 800 mM KCl. The cell suspension was centrifuged for 45 min at 100 000 g at 4°C. The in OGT-enriched supernatant was used for biochemical assays.

Cloning and expression of Nup62 in bacteria

Nup62 (the N-terminal 260 amino acids) was cloned from rat brain cDNA by PCR amplification with the following primer pair: Nup_F 5’-CACCATGCTCCCTCAGCTACCAGACAC3’ and Nup_R 5’-CTGCTACAGGGGACGACTGCTC3’. After amplification, the PCR product was ligated into pENTR/D-TOPO, the Nup62 fragment was shuttled using a gateway reaction into pDEST17 vector according to the manufacturer’s protocol (Invitrogen). N-terminal His-tagged Nup62 was expressed using BL21-AI bacteria. Cultures were grown at 37°C to mid-log phase and then induced with 0.2% of isopropylthio-β-D-galactoside for 2 h.

O-GlcNAc transferase assay

O-GlcNAc transferase reactions were performed with 0.2–μg of purified recombinant NFAT (BioSource) or Nup62 (0.5–10 μg) in the presence of 0.5 μCi UDP-[3H]GlcNAc, S9 lysate containing enriched OGT (see above) diluted 1:10 in 50 mM Tris–HCl, pH 7.5, 1 mM DTT and 12.5 mM MgCl2 and incubated for 30 min at 37°C. Glycosylated products were detected by two independent methods: (1) O-GlcNAc transferase reaction mixtures were separated on standard SDS–PAGE, the gels were fixed for 30 min in isopropanol:water:acetic acid (5:13.2) and incubated with Amplify (Amergram Biosciences) for 30 min prior drying. An autoradiography was performed for detection. The TCA method was performed as a second method (Marshall et al., 2003). Radioactivity was measured after addition of 5 ml scintillation fluid (OptiPhase HiSafe 3, PerkinElmer) in a liquid scintillation counter (2000 CA Tricarb, Packard).

RNA-mediated knockdown of OGT and Lck

5 × 10^5 Jurkat or BJAB cells were transfected by HiPerfect (QIAGEN) with a negative control or siRNA oligonucleotides specific for human OGT or Lck (OGT_1: ID11253, OGT_2: ID11254, OGT_3: ID670, Lck: 5019647, all purchased from Ambion). As a negative control, we used a nonsense siRNA, which is labeled with Alexa Fluor 488 (QIAGEN, 1022563). Alternatively, Amaxa transfections were performed using the solution V and program C16 according to the manufacturer’s protocol (Amaxa Biosystems). Transfected cells were incubated at 37°C for 24, 48 or 72 h before stimulation and further analysis. Transfection efficacy was evaluated by FACS where an aliquot of the cells treated with the nonsense siRNA Alexa Fluor 488 (QIAGEN, 1022563) was analyzed. Under the conditions used, we normally detected more than 90% of the cells positive for fluorescent signal, while only a small portion of the cells were dead (<10%).

Luciferase assays

A total of 500 ng of vector (GFP or OGT) was transfected together with 1000 ng of the NFκB-luciferase construct pLuc-4xNF-κB (Proksch et al, 2005) in 10^5 BJAB cells. Luciferase assays with Jurkat cell stably transfected with NFAT, IL-2 promoter- and NFκB-reporter plasmid were performed with 10^6 cells per experiment. The cells were stimulated as described in the legend to the Figures and subsequently lysed for 20 min at room temperature in 50 μl lysis buffer (passive lysis buffer; Promega), followed by centrifugation (10 000 g) for 20 min to sediment insoluble materials. A total of 5 μl of cell lysates was mixed with 50 μl of the luciferase assay mixture (470 pM Beetle Luciferin (Promega), 1.07 mM (MgCO3)2·Mg(OH)2 · H2O, 20 mM N-Tris-(hydroxymethyl)-methyl-glycine, 2.67 mM MgSO4, 100 μM EDTA, 33.3 mM DTT, 270 μM CoA(OAc), and 530 μM ATP), and relative light units were measured with a duoluminomat (Victor, Perkin Elmer).

Acknowledgements

We thank Dr Inna Lavrik for critically reading the manuscript and Dr Min Li-Weber for kindly providing the NFκB-luciferase reporter plasmid.

Conflict of interest

The authors have no conflicting financial interests.

References


4378 The EMBO Journal VOL 26 | NO 20 | 2007 ©2007 European Molecular Biology Organization


