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Stimulation of TK1 Lymphoma Cells via $\alpha_4\beta_7$ Integrin Results in Activation of src-Tyrosine- and MAP-Kinases

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The lymphocyte integrin $\alpha_4\beta_7$ is a cell surface adhesion receptor involved in initiating lymphocyte homing to gut-associated/mucosal lymphoid tissues by binding the mucosal addressin cell adhesion molecule-1 (MAdCAM-1). Other known ligands are vascular cell adhesion molecule-1, fibronectin, and the α_4 integrin chain itself. Here, we demonstrate that stimulation of the $\alpha_4\beta_7$ integrin through its α_4 subunit (mAb R1-2), β_7 subunit (mAb M293), or the combinatory epitope (mAb DATK32) enhances tyrosine phosphorylation of several cellular proteins in the murine TK1 lymphoma cell line. The two src-kinases p56lck and p59fyn were identified as possible mediators and substrates of the detected tyrosine phosphorylation. Furthermore, we observed activation of the MAP-kinases ERK1/2. © 1997 Academic Press

Integrins are a widely distributed family of heterodimeric transmembrane glycoproteins, mediating adhesion between cells and with their extracellular matrix (9).

The α_4 integrin is able to associate to $\alpha_4\beta_7$ (10) or $\alpha_4\beta_7$ (11, 12). Both α_4 integrins are implicated in adhesion to endothelial cells during lymphocyte homing (2, 3) and fetal and adult hematopoesis of T and B cells (13). Ligands for both α_4 integrins are FN, VCAM-1 and the α_4 chain itself (5-8). Only $\alpha_4\beta_7$ binds specifically to the MAdCAM-1 receptor molecule, a member of the Immunoglobulin superfamily (1, 12).

Recirculation and homing of leukocytes to lymphoid tissues is critical for the regulation of the immune response (14-16). Homing to mucosal tissues of the gut like Peyer's patches is mainly caused by $\alpha_4\beta_7$ integrin and to a much lesser extent by L-selectin binding to MAdCAM-1 (1-4, 17). It is characterized by a multistep cell adhesion process between adhesion molecules on the surface of leukocytes with their endothelial ligands, resulting in reversible rolling and tethering under shear flow as a prerequisite for firm adhesion and extravasation of leukocytes (1-3, 17).

Recently, we and others have identified intracellular signaling events upon L-selectin triggering in leukocytes (18-22). We detected in Jurkat T lymphocytes an activation of the Ras/MAP-kinase pathway dependent on the activation of p56lck kinase (20). As downstream effects of the Ras activation we found an L-selectin induced actin polymerization (21), activation of JNK (22) and synthesis of reactive oxygen intermediates (20).

Integrins transduce signals from outside to the intracellular space and vice versa, but so far, no intrinsic enzymatic activity of the integrin heterodimers has been identified (23).

Cellular stimulation via integrins results in several signaling events, e.g. association of c-src, the adapter protein Grb2 and p125FAK, followed by activation of MAP-kinases (24). Further, multiple tyrosine phosphorylated proteins were detected early after triggering via $\alpha_3\beta_7$ or $\alpha_4\beta_1$ (25-28). Likewise, β_7 crosslinking on human tonsillar B cells and on a B cell line enhances tyrosine phosphorylation of several proteins, including p125FAK (28). However, little is known about signaling induced by $\alpha_4\beta_7$ integrin in T lymphocytes.

In the present study, we investigated intracellular signaling events via $\alpha_4\beta_7$ integrin triggering, which shares functional similarities to L-selectin in homing of lymphocytes to lymphoid organs (17, 29).

We demonstrate that stimulation of the $\alpha_4\beta_7$ integrin in the murine $CD8^+$ T cell lymphoma cell line TK1 with monoclonal antibodies (mAb) against the α_4 subunit, the β_7 subunit or both subunits results in enhanced

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tyrosine phosphorylation of several proteins. In addition, the src-kinases p56lck and p59fyn as well as ERK1/2 are activated upon $\alpha_4\beta_7$ integrin triggering pointing to a signaling cascade from $\alpha_4\beta_7$ via src-kinases to ERK1/2.

METHODS

Antibodies. Anti-α_4 mAb (clone R1-2), anti-β_7 mAb (clone M293) or anti-β_1 mAb (clone 9EG7), were purchased from Pharmingen (San Diego, CA), mAb DATK32 was kindly provided by Dr. P. Altevogt (6), rabbit polyclonal anti-ERK1/2, rabbit polyclonal anti-p59fyn, rabbit polyclonal anti-p56lck and anti-phosphotyrosine antibody (4G10) were obtained from Upstate Biotechnology Inc. (UBI, Lake Placid, NY).

Cell culture. All reagents were purchased from Sigma (Deisenhofen, Germany) if not otherwise cited. Mouse TK1 lymphoma cells (a kind gift by Dr. B. Holzmann) were maintained in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS), 10 mM HEPES (pH 7.3), 2 mM L-glutamine, 1 mM sodium pyruvate, 100 μM nonessential amino acids, 100 U/ml penicillin, 100 μg/ml streptomycin, and 50 μM β-mercaptoethanol (complete RPMI-1640), all purchased by Gibco BRL, Eggenstein, Germany.

Stimulation and lysis. Cells (2-5$\times10^6$/sample for whole cell lysates (in 20 μl), 20$\times10^6$/sample for immunoprecipitation (in 100 μl) and 5$\times10^6$/sample for in vitro kinase assays (in μl) were washed twice and resuspended in HEPES/Saline, pH 7.3 (H/S, 132 mM NaCl, 20 mM Hepes, 5 mM KCl, 1 mM $CaCl_2$, 0.7 mM $MgCl_2$, 0.8 mM $MgSO_4$) and were activated at 37°C with anti-α_4-, anti-β_7-antibodies or DATK32, respectively. After the indicated time periods stimulation was terminated by lysis in a buffer containing 25 mM HEPES (pH 7.3), 125 mM NaCl, 0.1% SDS, 0.5% deoxycholate, 1% TritonX-100, 10 mM each of NaF, Na_3VO_4, sodium pyrophosphate and protease inhibitors (20 μg/ml of each aprotinin and leupeptin). Lysates were centrifuged at 25.000×g for 15 min at 4°C and supernatants were subjected to immunoprecipitation.

Immunoprecipitation. Cell lysates were incubated with 3 μg of specific antibodies or irrelevant control antibodies for 4 h at 4°C, followed by addition of 50 μl Pansorbin (Calbiochem, San Diego, CA) for MAP-kinase or 25 μl protein-A/G coupled agarose (Santa Cruz Inc., CA, USA) for 1 h at 4°C. The immunoprecipitates were washed 6-times in lysis buffer, boiled for 5 min in reducing SDS-sample buffer (60 mM Tris (pH6.8), 2.3% SDS, 10% glycerol, 5% β-mercaptoethanol), and subjected to SDS polyacrylamide gel electrophoresis (SDS/PAGE).

Immunoblotting. Whole cell lysates or immunoprecipitated proteins were separated by 10% SDS/PAGE under reducing conditions, transferred electrophoretically to Nitrocellulose membranes (Bio-Rad, Munich, Germany), membranes were blocked with 4% BSA in 1×TBS-Tween and incubated for 4 h at 4°C with specific antibodies in 1xTBS-Tween. Immunoreactive bands were visualized by using secondary horseradish peroxidase-conjugated protein G (BioRad, Munich, Germany) and a chemoluminescence method (Amersham, Braunschweig, Germany). All blots were stripped after analysis by incubation in 20 mM Tris (pH6.8), 2% SDS, 70 mM β-mercaptoethanol at 70°C for 45 min and reprobed to test for equal amounts of immunoprecipitated proteins.

In vitro kinase assay. ERK1/2 immunoprecipitates were washed 3-times in each of lysis buffer and kinase buffer, resuspended in

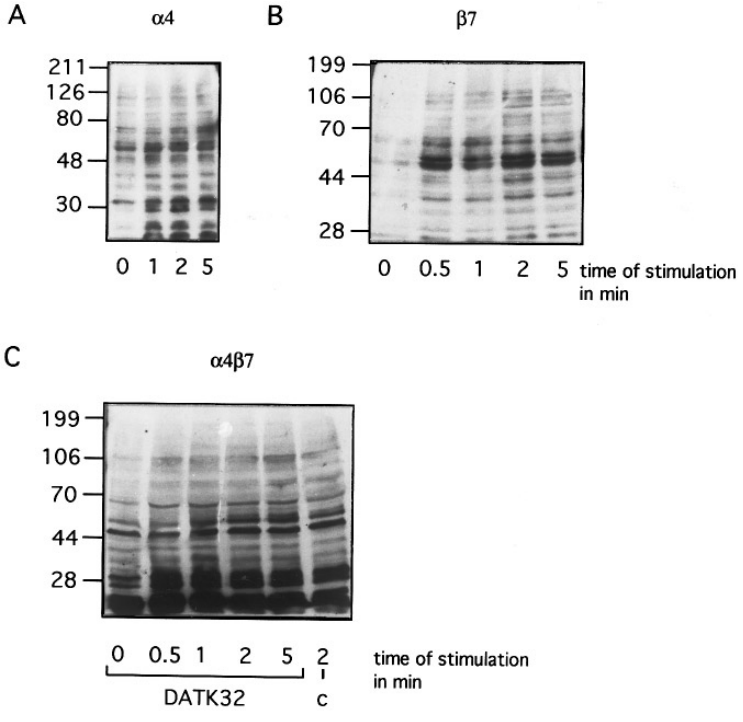

FIG. 1. α_4 ligation by mAb R1-2 (A), β_7 ligation by mAb M293 (B) or $\alpha_4\beta_7$ ligation by DATK32 (C) induces tyrosine phosphorylation of multiple proteins. TK1 cells were stimulated for different times with the indicated antibodies, lysed, separated by SDS/PAGE and immunoblotted with the anti-P-Tyr antibody 4G10. As a control (c), TK1 cells were stimulated with their RPMI medium supernatant for 2 min. The results are representative of three blots.

kinase buffer, containing 25 mM HEPES (pH 7.4), 2 mM DTT, 10 mM $MgCl_2$, 10 μM ATP and 5 μCi of [γ-^{32}P]-ATP (NEN, DuPont, Germany) for 15 min at 37°C. An aliquot was analyzed by Western blotting for equal amounts of MAP-K in each lane. For p56lck and p59fyn activity immunoprecipitates were resuspended in a kinase buffer of 25 mM HEPES (pH 7.3), 0.15 M NaCl, 10 mM $MgCl_2$, 0.5% NP40, 1 mM Na_3VO_4, 5 mM DTT, 10 μm ATP, and 1 μCi [γ-^{32}P]-ATP, incubated at 30°C for 15 min. Samples were separated by SDS/PAGE, transferred electrophoretically to membranes and visualized by autoradiography. The intensity of the bands was evaluated by densitometric scanning using a conventional desktop scanner and the NIH Image (version 1.61). Measurements were corrected by subtraction of the stimulated, but non-specific immunoprecipitated control (up).

Flow cytometry. To confirm expression of $\alpha_4\beta_7$ integrin, TK1 cells (2×10^6/sample) were washed twice in H/S supplemented with 2% FCS and 0.2% NaN_3, resuspended in the same buffer, incubated for 45 min at 4°C with 2 μg/ml anti-α_4-antibodies, anti-β_7-antibodies or anti-β_1-antibodies. Cells were washed then twice in H/S, 2% FCS and 0.2% NaN_3, stained with a FITC-labeled anti-rat-Ig (Sigma) for 45 min at 4°C, washed twice again and analyzed for binding anti-α_4, anti-β_1- or anti-β_7-antibodies by flow cytometry using a FACSort (Becton-Dickinson, Heidelberg, Germany).

RESULTS

Ligation of α_4, β_7 or $\alpha_4\beta_7$ induces cellular tyrosine phosphorylation in TK1 lymphoma cells. Stimulation of TK1 cells using anti-α_4 mAb (clone R1-2) or anti-β_7 mAb (clone M293) resulted in a rapid tyrosine phosphorylation of proteins with a molecular weight of approximately 30, 45, 55, 60, 70, 80 and 110 kDa (Fig. 1A, B). Crosslinking α_4 or β_7 with a secondary anti-rat antibody did not enhance the detected increase in tyrosine phosphorylation (data not shown).

Ligation of $\alpha_4\beta_7$ by DATK32, an antibody that is directed against a combinatory epitope of $\alpha_4\beta_7$, also initiated tyrosine phosphorylation of several proteins (Fig. 1C). To confirm specificity of stimulation via DATK32 we treated cells for 2 min with RPMI medium supernatant from TK1 cells (c in Fig. 1C) which showed less tyrosine phosphorylation. Therefore, integrin activation by antibodies directed against the α_4 or β_7 subunit or the $\alpha_4\beta_7$ dimer induces tyrosine phosphorylation of several cellular proteins.

Src-kinase activity is enhanced after α_4 and $\alpha_4\beta_7$ ligation. Since src-kinases play an important role in early signaling events in leukocytes, we tested for activation of p59fyn and p56lck by examining their in vitro autophosphorylation. Integrin triggering using anti-α_4 mAb R1-2 or anti-$\alpha_4\beta_7$ mAb DATK32 induces an increase in

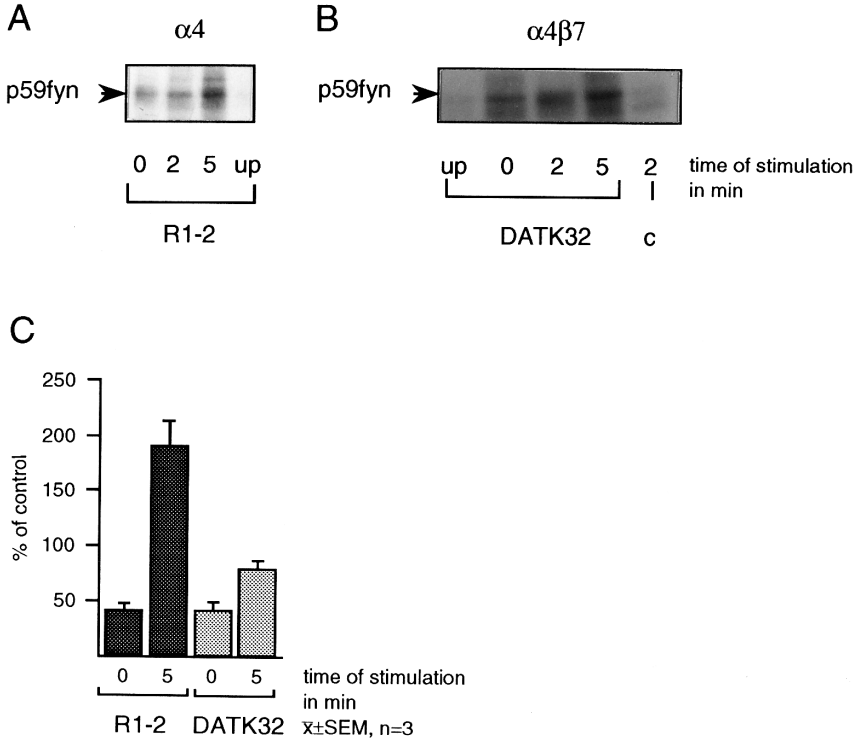

FIG. 2. The src-like kinase p59fyn is activated by integrin stimulation via α_4 (R1-2) (A) and by a combinatory epitope on $\alpha_4\beta_7$ (DATK32) (B). TK1 lysates were immunoprecipitated with anti-p59fyn antibodies (A, B) and kinase activity was measured in an in vitro assays. The controls, a 2-min-stimulated, but non-specific immunoprecipitate (up) and cellular treatment of TK1 cells with their supernatant (c) do not show increased kinase activity. Laser densitometry of the blots revealed a 5-fold increase of p59fyn activity after mAB R1-2, whereas incubation using the DATK32 antibody results in a 2-fold increase (C). Data are corrected by subtraction of the non-stimulated, but immunoprecipitated sample (up) and are representative of three blots.

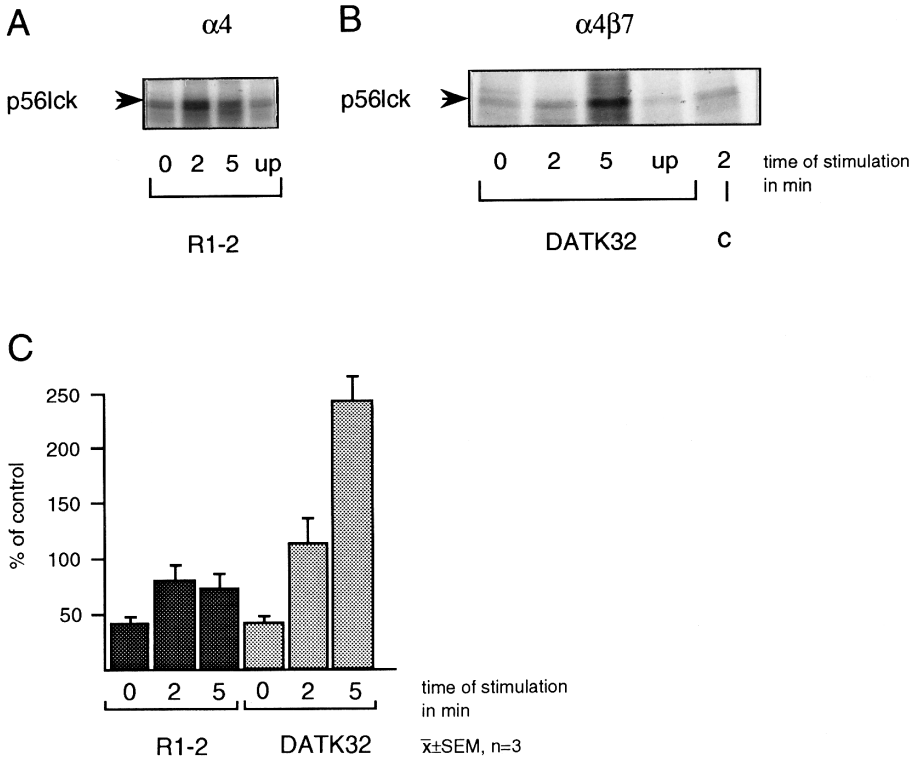

FIG. 3. Integrin stimulation via α_4 (mAb R1-2) (A) or $\alpha_4\beta_7$ (DATK32) (B) induces activation of p56lck detected by in vitro kinase assays. As evaluated by densitometric scanning R1-2 or DATK32 elevate kinase activity of p56lck 2-fold or 6-fold, respectively (C). Data are representative of three blots.

p59fyn activity after 2 and 5 min of stimulation (Fig. 2A, B), whereas a 2-min-stimulated non-specific immunoprecipitated sample (up) as well as 2-min-incubation with the centrifuged medium supernatant from TK1 cells (c) did not lead to activation of p59fyn. Densitometric scanning evaluated the increase of basal levels of p59fyn activity as 5-fold after R1-2 stimulation and 2-fold after DATK32 stimulation (Fig. 2C). In addition, we tested the activity of p56lck after α_4 or $\alpha_4\beta_7$ ligation (Fig. 3). The in vitro kinase activity of p56lck is also enhanced time-dependently, but resulted in a 2-fold increase in phosphorylation induced by R1-2 ligation compared to a 6-fold increase upon DATK32 stimulation (Fig. 3A-C). These data imply a role for p56lck and p59fyn in signal transduction after $\alpha_4\beta_7$ integrin triggering.

Tyrosine phosphorylation and kinase activity of ERK1/2 following α_4 and β_7 ligation. Next, we tried to identify possible downstream signaling events of src-like kinases. The results displayed in Fig. 1 indicate an increase in tyrosine phosphorylation of proteins with a molecular weight of about 42-45 kDa pointing to a possible tyrosine phosphorylation of MAP-kinases.

The MAP-kinases ERK1/2 were immunoprecipitated from TK1 cells stimulated with anti-α_4 or anti-β_7 and evaluated for tyrosine phosphorylation. The results show an increase of ERK1/2 tyrosine phosphorylation after both stimuli (Fig. 4A, B). Membranes were reprobed with anti-ERK1/2 antibodies to test for equal amounts of immunoprecipitated proteins (Fig. 4A, B, lower part). MAP-kinases are known to be activated by phosphorylation on tyrosine and serine/threonine residues. To confirm our results from the Western blotting, we examined ERK1/2 activity in an in vitro kinase assay (Fig. 4C). α_4 and β_7 ligation induced an activation of ERK1/2 with a faster and stronger increase in phosphorylation after α_4 triggering. Aliqouts of the same samples were probed with anti-ERK1/2 antibodies to test for equal amounts of protein (Fig. 4C). These results indicate that $\alpha_4\beta_7$ integrin ligation leads to activation of the MAP-kinases ERK1/2.

DISCUSSION

Homing receptors like $\alpha_4\beta_7$ integrin and L-selectin mediate selective transient interactions between lymphocytes and specialized endothelial cells to direct subsets of lymphocytes to lymphoid organs, where they emigrate and recirculate (14-16). Both adhesion molecules recognize at different epitopes the endothelial li-

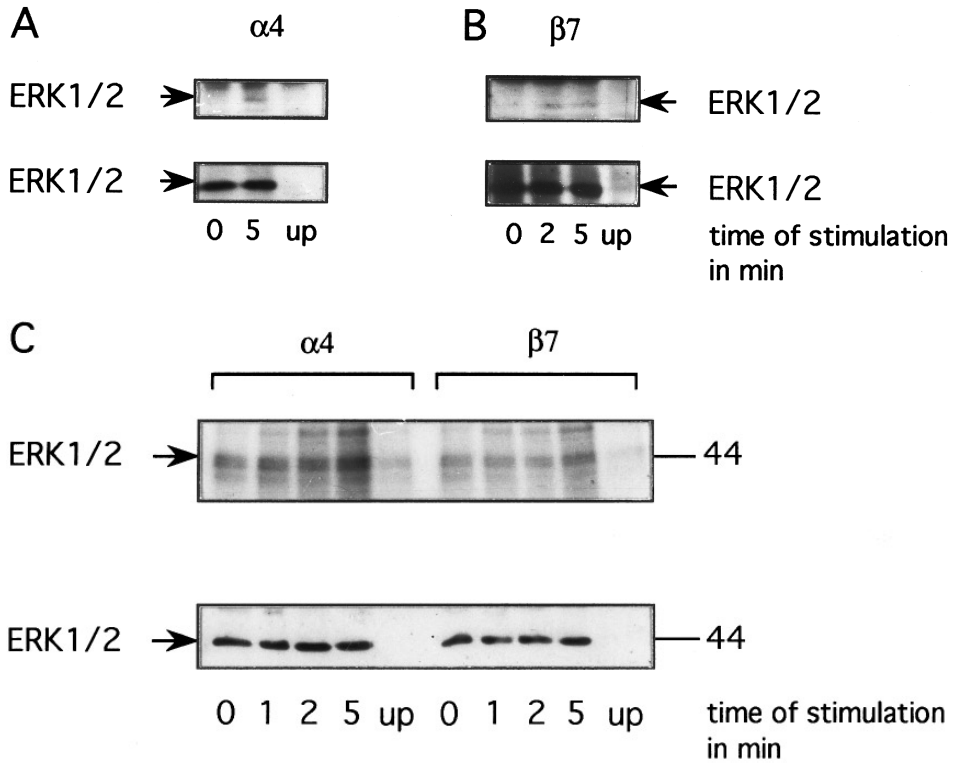

FIG. 4. α_4 (A) or β_7 (B) ligation leads to tyrosine phosphorylation of MAP-kinase. Anti-ERK1/2 immunoprecipitates were analyzed by Western blotting with the anti-phosphotyrosine antibody 4G10 (upper panel). Membranes were reprobed with anti-ERK1/2 antibodies to check for equal amounts of protein in each lane (lower panel). MAP-kinase immunoprecipitates, either stimulated with anti-α_4 mAb or anti-β_7 mAb were tested for their in vitro kinase activity (C). Control immunoprecipitation from 2min stimulation, but non-specific immunoprecipitation shows neither increased phosphotyrosine content (A, B) nor kinase activity (C).

gand MAdCAM-1 expressed on mucosal lymphoid tissue (1-3, 17).

Recently, we and others have identified a signaling function of the L-selectin molecule (18-22). In Jurkat T lymphocytes we demonstrated an activation of the Ras/MAP-kinase pathway depending on the activation of p56lck kinase (20). Furthermore, we detected as downstream effects of Ras activation actin polymerization (21), activation of JNK (22) and synthesis of reactive oxygen intermediates (20).

Here, we report intracellular signaling events upon $\alpha_4\beta_7$ integrin triggering in murine TK1 lymphoma cells. Using antibodies that recognize either α_4 (mAb R1-2), β_7 (mAb M293) or a combinatory epitope on $\alpha_4\beta_7$ (mAb DATK32) we detected an increase in tyrosine phosphorylation of several intracellular proteins in whole cell lysates, activation of the two src-like kinases p56lck and p59fyn and activation of the MAP-kinases ERK1/2.

The increase in tyrosine phosphorylation through the α_4 subunit or the β_7 subunit could not be enhanced by crosslinking of α_4 or β_7 with a secondary antibody (data not shown). However, proteins of about 30-33 kDa and about 25-28 kDa show elevated tyrosine phosphorylation after α_4 ligation (R1-2) and $\alpha_4\beta_7$ (DATK32) ligation in contrast to β_7 (M293) ligation. This may reflect the tendency that stimulation of the $\alpha_4\beta_7$ integrin heterodimer with different antibodies causes different signaling events.

A panel of antibodies directed against $\alpha_4\beta_7$ has been reported, identifying different epitopes on $\alpha_4\beta_7$ (6). The anti-α_4 mAb R1-2 is characterized by inhibition of adhesion to VCAM-1 or FN, but does not affect interaction with MAdCAM-1 (1, 6, 11). DATK32 recognizes a combinatory epitope on $\alpha_4\beta_7$, that is involved in $\alpha_4\beta_7$ mediated adhesion to VCAM-1, FN and MAdCAM-1 (4, 6). After stimulation with R1-2 or DATK32 we observed activation of the src-kinases p59fyn and p56lck. As evaluated by densitometric scanning R1-2 induced a stronger increase of basal level in p59fyn activity (5-fold) than in p56lck activity (2-fold). In contrast, DATK32 ligation increased p56lck activity 6-fold, but it elevated p59fyn activity only 2-fold. This implies that stimulation of these different epitopes activates the same tyrosine kinases with different intensities. It suggests that interaction with different ligands, that bind to distinct epitopes on the $\alpha_4\beta_7$ heterodimer may modulate intracellular signaling. It will be of future interest

to confirm these results obtained with antibodies by triggering $\alpha_4\beta_7$ with its natural ligands.

Since integrins lack any known intrinsic kinase activity (23), an association of the cytoplasmic domain with signal transducing molecules is necessary. Recently, an association of the short cytoplasmic domain of the β_7 subunit with p125FAK (28, 30), resulting in activation of and phosphorylation on p125FAK Tyr-397, association with src-like tyrosine kinases and assembling focal adhesion complexes has been shown (31-32). Since our preliminary results show a similar phophorylation of FAK in T cells after $\alpha_4\beta_7$ integrin ligation, it might be possible that p125FAK mediate the activation of p56lck and p59fyn.

Tyrosine phosphorylation of MAP-kinases has been described to be mediated by an association of p125FAK, Grb2 and Sos resulting in p21Ras activation, a known ERK1/2 regulator (24, 33). Since the activation of H-Ras and its kinase effectors Raf-1 as well as ERK1/2 seems to inhibit integrin function the activation of ERK1/2 upon $\alpha_4\beta_7$ integrin ligation may function as a negative feedback loop for $\alpha_4\beta_7$ integrins (34).

In summary, our experiments show that $\alpha_4\beta_7$ triggering by antibodies detecting different epitopes results in tyrosine phosphorylation of several cellular proteins and activation of p56lck, p59fyn as well as ERK1/2, implying a role of these molecules for outside-in signaling of $\alpha_4\beta_7$ integrins in TK1 cells.

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