Characterization of β_2 (CD18) integrin phosphorylation in phorbol ester-activated T lymphocytes

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Integrins are transmembrane proteins involved in cell–cell and cell–extracellular-matrix interactions. The affinity and avidity of integrins for their ligands change in response to cytoplasmic signals. This ‘inside-out’ activation has been reported to occur also with β_2 integrins (CD18). The β_2 integrin subunit has previously been shown to become phosphorylated in T lymphocytes on cytoplasmic serine and the functionally important threonine residues after treatment with phorbol esters or on triggering of T-cell receptors. We have now characterized the phosphorylation of β_2 integrins in T-cells in more detail. When T-cells were activated by phorbol esters the phosphorylation

was mainly on Ser⁷⁵⁶. After inhibition of serine/threonine phosphatases, phosphorylation was also found in two of the threonine residues in the threonine triplet 758–760 of the β_2 cytoplasmic domain. Activation of T-cells by phorbol esters resulted in phosphorylation in only approx. 10 % of the integrin molecules. Okadaic acid increased this phosphorylation to approx. 30 % of the β_2 molecules, assuming three phosphorylation sites. This indicates that a strong dynamic phosphorylation exists in serine and threonine residues of the β_2 integrins.

Key words: adhesion, phosphatases, protein kinases.

INTRODUCTION

Integrins are transmembrane proteins that form a superfamily of proteins involved in regulated cell–cell and cell–substratum interactions [1]. They have a prominent role in the attachment and detachment of cells to/from their surrounding matrix and in a number of different adhesion events in the immune system. All integrins discovered so far are heterodimers consisting of α and β subunits. The family was originally characterized by structurally related β subunits that formed a series of heterodimers with distinct α subunits. However, more recent findings indicate that there are several important exceptions to this concept.

The affinity or avidity of integrins for their extracellular ligands can change in response to cytoplasmic signals initiated by the stimulation of cellular receptors [2,3]. This ‘activation’ of integrins has been termed ‘inside-out’ signalling [4,5]. Several integrin families have been shown to undergo activation, such as the β_3 -integrins in platelets [4], the β_2 -integrins in leucocytes [3] and the β_1 -integrins in haemopoietic cells [6]. In addition to the affinity modulation the integrins also activate intracellular signalling pathways in a process termed ‘outside-in’ signalling [7]. These signals produced by engagement of integrins include the generation of lipid second messengers, the activation of protein kinases, phosphoinositide kinases and small GTP-binding proteins and changes in intracellular pH and [Ca²⁺].

Integrin β subunit cytoplasmic domains have a central role both in ‘inside-out’ and ‘outside-in’ signal transduction [8]. The cytoplasmic domain sequences are highly conserved between species, which suggests an indispensable role for these portions of the polypeptides. A number of elements in the cytoplasmic domains of integrin β subunits have been shown to be important both in the regulation of integrin activity [5] and in the signal

transduction mediated by integrins [8]. These include the membrane-proximal elements in the β_1 and β_3 polypeptides whose deletion leads to a constitutively active phenotype [9]. The cytoplasmic portions contain binding sites for cytoskeletal proteins such as α -actinin [10], talin [11] and paxillin [12] and for regulatory proteins such as β_3 -endonexin [13]. Particularly important are the Asn-Pro-Xaa-Tyr regions implicated in the activation of β_1 and β_3 integrins [14] and the Thr-Thr-Thr triplet necessary for the adhesion mediated by β_2 integrins [15].

The cytoplasmic domains of the integrin β subunits contain a number of putative phosphorylation sites. Because integrin phosphorylation might be a mechanism by which both integrin activation and signal transduction could be regulated, the phosphorylation status of integrins has been extensively studied. β_1 integrin [16] and β_3 integrin [17,18] have been shown to become phosphorylated on tyrosine residues; this tyrosine phosphorylation is likely to take place in their conserved Asn-Pro-Xaa-Tyr motifs. In addition, the β_4 integrin subunit has been shown to become tyrosine-phosphorylated [19]. These tyrosine phosphorylation events are generally induced by cell adhesion and are thus believed to mediate ‘outside-in’ signal transduction by integrins.

Phorbol esters, potent activators of protein kinase C, have been found to induce β_2 integrin activation in leucocytes [20,21] as well as β_1 integrin activation of haemopoietic cells [6]. The integrin $\alpha_{\text{L}}\beta_3$, which can normally be stimulated by agonists such as thrombin or platelet-activating factor in platelets [4], can also be activated by phorbol esters [22]. These findings led to speculation on the involvement of serine/threonine phosphorylation in the ‘inside-out’ signalling of integrins. In β_3 the phosphorylation was shown to occur on threonine residues only after stimulation by thrombin or phorbol ester [23].

Abbreviations used: FCS, fetal calf serum; PDBu, phorbol 12,13-dibutyrate; TCR, T-cell receptor.

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The β_2 (CD11/CD18) integrins comprise a family of leucocyte integrins with four distinct α chains (α_L or CD11a, α_M or CD11b, α_X or CD11c and α_D or CD11d) [24]. They are expressed solely on leucocytes and are of pivotal importance in leucocyte adhesion. The activation of β_2 integrin can be triggered artificially by phorbol esters [20,21], and with antibodies directed against β_2 integrins [25,26] or other leucocyte surface proteins such as the T-cell receptor (TCR) complex [27]. The mechanism by which this 'inside-out' activation is mediated is poorly understood. The phosphorylation of leucocyte integrins has been studied extensively and the phosphorylation state of β_2 integrins has been reported to change in phorbol ester-activated leucocytes [28–31]. The α chains were shown to be constitutively phosphorylated, whereas the β_2 chain was reported to become phosphorylated only after treatment with phorbol ester. The induced phosphorylation of β_2 occurred almost exclusively on serine [29,31], and in the study where the putative phosphorylation sites of β_2 were mutated, the major phosphorylation site was identified as Ser⁷⁵⁶ in the β_2 cytoplasmic domain [15]. Although activation by phorbol esters revealed only serine phosphorylation in β_2 under these experimental conditions, it has become evident that the activation also induces a dynamic threonine phosphorylation in β_2 , which can be revealed only when serine/threonine phosphatases in T-cells are inhibited by okadaic acid [32].

The amino acids on which the rapid phosphorylation/dephosphorylation of β_2 takes place are not known, but the only threonine residues present in the β_2 cytoplasmic domain exist in the threonine triplet (758–760) shown to be vital for the activation of the integrin [15]. We therefore considered it important to map the dynamic phosphorylation sites in β_2 .

EXPERIMENTAL

Materials

RPMI 1640, L-glutamine and penicillin/streptomycin were purchased from Hyclone Europe Ltd (Cramlington, Northumberland, U.K.). Fetal calf serum (FCS) was obtained from Biological Industries (Kibbutz Bet HaEmak, Israel). Phosphate-free minimal essential medium, PMSF, aprotinin, leupeptin, iodoacetamide, quercetin, tosyl-lysylchloromethane ('TLCK'), tosylphenylalanylchloromethane ('TPCK'), *N*-benzyloxycarbonyl-L-phenylalanine chloromethyl ketone, polyvinylpyrrolidone and glucose were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). ATP, ADP, AMP, GTP and GDP were obtained from Boehringer Mannheim (Mannheim, Germany) and an ATP-monitoring reagent from BioOrbit (Turku, Finland). Ninhydrin was obtained from Merck (Darmstadt, Germany). Protein G–Sepharose and Ficol-Hypaque were from Pharmacia Biotech (Uppsala, Sweden). Sequencing-grade modified trypsin (activity more than 2.5 units/mg) was purchased from Promega (Madison, WI, U.S.A.) and hexokinase (1500 units/ml) from Boehringer Mannheim. Phorbol 12,13-dibutyrate (PDBu) (Sigma) and okadaic acid (Calbiochem-Novabiochem Co., La Jolla, CA, U.S.A.) were dissolved in DMSO. [³²P]P_i (aqueous solution; 10 mCi/ml, 5000 Ci/mmol) was purchased from the Radiochemical Centre (Amersham, Little Chalfont, Bucks., U.K.). The monoclonal antibody R7E4 against the β_2 subunit of leucocyte integrins has been described previously [33].

Synthesis of peptides

Synthetic peptides were synthesized by solid-phase synthesis using 9-fluorenylmethoxycarbonyl chemistry in an Applied Biosystems model 433A automatic peptide synthesizer. Amino acids

were activated by using the 2-(1-*H*-benzotiazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate/1-hydroxybenzotriazole method. Peptides were purified by HPLC with a C₁₈ reverse-phase column (Bondapack; Waters) and the sequences were confirmed by matrix-assisted laser desorption ionization–time-of-flight MS mass analysis. The following β_2 cytoplasmic phosphopeptides were synthesized: 24-residue peptides (residues 746–769) with either Ser⁷⁵⁶ (S⁷⁵⁶-peptide), Ser⁷⁵⁶ and Thr⁷⁵⁸ (S⁷⁵⁶T-peptide), Ser⁷⁵⁶ and Thr⁷⁵⁸–Thr⁷⁵⁹ (S⁷⁵⁶T2-peptide) or Ser⁷⁵⁶ and Thr⁷⁵⁸–Thr⁷⁵⁹–Thr⁷⁶⁰ (S⁷⁵⁶T3-peptide) phosphorylated, a nine-residue peptide (residues 725–733) with Ser⁷³⁰ (S⁷³⁰-peptide) phosphorylated, an 11-residue peptide (residues 745–755) with Ser⁷⁴⁵ phosphorylated (S⁷⁴⁵-peptide) and a 4-residue peptide otherwise corresponding to residues 766–769 except Glu⁷⁶⁸ and phosphorylated Ser⁷⁶⁹ switched (S⁷⁶⁹-peptide), because the synthesis was more easily accomplished when the phosphorylated serine residue was not at the C-terminus.

T-cell isolation

Buffy coats used for the isolation of T-cells were obtained from the Finnish Red Cross Blood Transfusion Service (Helsinki, Finland). Mononuclear leucocytes were isolated by Ficol-Hypaque gradient centrifugation. T-cells were further enriched by using nylon-wool columns. Enriched T-cells were either subjected to radiolabelling with ³²P or suspended in RPMI 1640 medium supplemented with 10% (v/v) FCS, 1% (w/v) L-glutamine and antibiotics and 10 mM Hepes and left in culture overnight at 37 °C.

Radiolabelling with ³²P and cell activation

Enriched T-cells were washed with phosphate-free minimal essential medium supplemented with 5% (v/v) dialysed FCS, 10 mM Hepes, 1% L-glutamine and antibiotics, then suspended in the same medium to a density of 50 × 10⁶ cells/ml. After preincubation for 1 h at 37 °C, 1–2 mCi of [³²P]P_i was added. Cells were labelled either overnight at 37 °C or for different durations as indicated.

The cells were preincubated with 1.5 μM okadaic acid for 25 min and subsequently activated with 200 nM PDBu for 30 min at 37 °C. The activation was stopped by the addition of ice-cold 10 mM EDTA/PBS, and the cells were washed once with ice-cold 2 mM EDTA/PBS. The cells were lysed at 0 °C for 15 min in 1% (v/v) Triton X-100/10 mM sodium phosphate/10 mM sodium pyrophosphate/50 mM sodium fluoride/1 mM sodium orthovanadate/10 mM EDTA/350 mM NaCl/1 mM PMSF/10 μg/ml aprotinin/10 μg/ml leupeptin/5 mM iodoacetamide/0.1 mM quercetin/0.1 mM tosyl-lysylchloromethane/0.1 mM tosylphenylalanylchloromethane/0.1 mM *N*-benzyloxycarbonyl-L-phenylalanine chloromethylketone (pH 7.4). Cell lysates were centrifuged at 60 000 *g* for 30 min at 2 °C and the supernatants were used either for immunoprecipitation or for the extraction of nucleotides with 1 vol. of EDTA/ethanol [10 mM EDTA in 86% (v/v) ethanol, pH 7.4].

Immunoprecipitation

Cell lysates were precleared with Protein G–Sepharose for 1 h at 4 °C; the β_2 integrin complexes were then immunoprecipitated with monoclonal antibody R7E4 covalently coupled [34] to Protein G–Sepharose. After incubation for 2.5 h at 4 °C with constant rolling, Sepharose beads were washed eight times with decreasing detergent and salt concentrations. Bound proteins were eluted with 1% (w/v) SDS at 40 °C for 40 min and then subjected to SDS/PAGE.

SDS/PAGE and silver staining

SDS/PAGE [8% (w/v) gel] was performed by the method of Laemmli [35]. For phosphopeptide mapping the samples were alkylated with 10 mM dithiothreitol/3 mM EDTA, and 20 mM iodoacetamide was added for 15 min at room temperature before SDS/PAGE.

Electrophoresed proteins were sometimes revealed by staining with silver. The gel was fixed in 30% (v/v) ethanol/0.5% (v/v) acetic acid for 1 h. After being washed, the gel was sensitized with 0.02% sodium thiosulphate for 1 min. The gel was rinsed, incubated with 0.2% silver nitrate for 30 min and developed in 37% (v/v) formaldehyde/3% (v/v) potassium carbonate/0.001% sodium thiosulphate.

Phosphopeptide mapping

For phosphopeptide mapping, ^{32}P -labelled proteins were resolved by SDS/PAGE, blotted on PVDF membranes (Millipore, Bedford, MA, U.S.A.), located with a Fuji BAS 1000 PhosphoImager (Tokyo, Japan) and the regions corresponding to β_2 were excised. The filter pieces were first saturated with polyvinylpyrrolidone 360 at 37 °C for 1 h; after being washed, the protein was digested with trypsin at 37 °C overnight. The resulting peptides were separated in the first dimension by electrophoresis at pH 8.0 by using the HTLE-7000 electrophoresis system (C.B.S. Scientific company, Del Mar, CA, U.S.A.) and in the second dimension by ascending chromatography [36]. ^{32}P -labelled peptides were detected and quantified by PhosphoImager analysis; synthetic peptides were revealed by ninhydrin staining.

Separation, identification and quantitation of nucleotides

^{32}P -labelled nucleotides and metabolites were separated by either TLC on 20 cm $\times$ 20 cm PEI-Cellulose F sheets (Merck, Darmstadt, Germany) with 1.2 M ammonium formate/0.8 M HCl or by two-dimensional paper chromatography on Whatman No. 1 paper as described [37]. The identities of the spots were verified with unlabelled standard ATP, ADP, AMP, GTP and GDP, which were detected by measuring A_{254} . The radioactivities in the spots were determined by liquid-scintillation counting (Wallac, Turku, Finland).

The amount of ^{32}P in the γ -phospho group of ATP was quantified by incubation of the T-cell lysates with 2 mM MgCl_2 /2 mM glucose/1 unit of hexokinase for 10 min at 37 °C, followed by 4 min in boiling water. Precipitated enzymes were centrifuged and the nucleotides were extracted and separated as described above.

Determination of the stoichiometry of phosphorylation

The stoichiometry of the phosphorylation of the β_2 subunit was analysed essentially as described [38], and calculated by comparing the incorporation of ^{32}P into β_2 from the γ -phospho group of [^{32}P]ATP; the amount of β_2 was measured by densitometry. In brief, T-cells were labelled with [^{32}P]P_i overnight, then activated and lysed as described. Adenine nucleotides in lysate were extracted and the ^{32}P incorporated into ATP was determined by liquid-scintillation counting after TLC. The absolute amount of ATP in lysates was measured by the luciferin/luciferase technique with an LKB Wallac 1250 luminometer (Turku, Finland) [39]. The ^{32}P incorporated into β_2 was determined after the integrin complexes had been immunoprecipitated and subjected to SDS/PAGE. Gels were stained with silver and the absolute amount of β_2 subunit was determined

by densitometric comparison with serial dilutions of purified β_2 molecules. For determination of the ^{32}P radioactivity in β_2 the specific area was cut out of the dried gels and heated for 1 h at 80 °C in 30% (v/v) H_2O_2 . The radioactivity was determined by liquid-scintillation counting.

RESULTS

Identification of the phosphorylation sites in the β_2 integrin subunit in phorbol ester-activated T-cells

Because the β_2 integrin subunit has previously been shown to become phosphorylated in phorbol ester-induced leucocytes both in a stable manner on a serine residue [15] and more dynamically on threonine residues [32], it was important to map the dynamic phosphorylation sites of β_2 . Tryptic phosphopeptide maps were made from ^{32}P -labelled T-cells (Figure 1) treated either with PDBu alone or with PDBu after pretreatment with okadaic acid. PDBu induced β_2 phosphorylation (Figure 1A), which was markedly augmented by preincubation with okadaic acid (Figure 1C). Okadaic acid alone did not induce β_2 phosphorylation (results not shown), as reported previously [32]. The phosphorylated β_2 was isolated and digested further with trypsin; the phosphopeptides produced were separated on cellulose plates (Figures 1B, 1D and 1E). Tryptic peptides digested from synthetic phosphopeptides comprising the last 24 residues at the C-terminus of the β_2 cytoplasmic tail with phosphate in either Ser⁷⁵⁶ or in different threonine residues 758–760 were added as markers (Figure 2).

In a phosphopeptide map of β_2 produced from PDBu-activated T-cells (Figure 1B) a spot was detected that migrated identically with the synthetic peptide having a single phosphorylation site on Ser⁷⁵⁶, the S⁷⁵⁶-peptide (indicated by S). Because it has been demonstrated previously that phosphorylation occurs only in

Figure 1 Tryptic phosphopeptides of the β_2 polypeptide

β_2 integrins were immunoprecipitated and subjected to SDS/PAGE from ^{32}P -labelled T-cells activated with either PDBu alone (A) or with PDBu after pretreatment with okadaic acid (C). β_2 was further digested with trypsin. The tryptic β_2 peptides obtained from T-cells with activation by PDBu alone (B) or with treatment with okadaic acid and PDBu together (D, E) were run on cellulose plates together with synthetic phosphopeptides (see Figure 2) also digested with trypsin. The experiments were repeated eight times with essentially similar results.

Figure 3 Saturation of ATP labelling with ^{32}P with time

T-cells were radiolabelled with ^{32}P for the indicated durations and after lysis the nucleotides were extracted. ^{32}P -labelled nucleotides were separated by TLC (A) and the radioactivities incorporated into ATP were determined (B). The results are representative of two separate experiments.

Figure 4 Quantification of ^{32}P in the γ -phospho group of ATP

Extracted nucleotides from lysates of ^{32}P -radiolabelled T-cells were separated by two-dimensional paper chromatography either without further treatment (A) or after treatment with hexokinase and glucose (B). The results are representative of two separate experiments.

Stoichiometry of β_2 phosphorylation

For the determination of the stoichiometry of β_2 phosphorylation it was necessary to determine the amount of radioactivity incorporated into the γ -phospho group of ATP. For this purpose we determined the saturation time of ATP synthesis in the ^{32}P -labelled T-cells (Figure 3). T-cells were labelled with [^{32}P]P_i for different durations and ATP was quantified from TLC plates (Figure 3A). The incorporation of ^{32}P into ATP continued to increase after labelling for 10 h; saturation of ATP with ^{32}P was achieved only after labelling overnight (Figure 3B). When two-dimensional paper chromatography of the extracted nucleotides from T-cells labelled overnight with ^{32}P was performed (Figure 4A), it became evident that radioactivity was not incorporated into the α -phospho group of ATP because there was no [^{32}P]AMP in the extracts. The migrations of ATP, ADP and AMP were verified by the addition of unlabelled standards. According to earlier chromatograms [37] the other spots represent P_i (indicated

Table 1 Determination of ^{32}P in ATP, ADP and ATP from radiolabelled T-cell lysates

The nucleotides extracted from ^{32}P -labelled lysates were subjected to two-dimensional paper chromatography followed by liquid-scintillation counting. The results are representative of two separate experiments. The AMP was unlabelled and was identified by UV absorption on the chromatogram. The last column shows the radioactivity gained (+) or lost (–) relative to the control after treatment with hexokinase and glucose (see Figure 4).

Chromatographic assignment of spot	Total radioactivity in control (d.p.m.)	Gain or loss in radioactivity (d.p.m.)
ATP	170644	–156948
ADP	56944	+ 82252
AMP	0	–

by y), phosphatidylethanolamine (indicated by z) and GTP and GDP (in the region indicated by x). When ^{32}P -labelled T-cell lysates were treated with hexokinase and glucose (Figure 4B), the decrease in radioactivity in the ATP spot was 92 %; 52 % of the radioactivity lost in the ATP spot was recovered in the spot for ADP (Table 1). Therefore the radioactivity of ATP from ^{32}P -labelled T-cells was present only in the β - and γ -phospho groups, and in 1:1 proportion.

Table 2 lists the results of the stoichiometry measurements. In Expts. 1–3, T-cells were activated with PDBu; the stoichiometry of β_2 phosphorylation obtained was between 0.11 and 0.13, yielding a mean stoichiometry of 0.12 mol of phosphate/mol of β_2 subunit. The results obtained with PDBu activation alone were highly reproducible. In contrast, the stoichiometry of β_2 phosphorylation after pretreatment with okadaic acid and activation by PDBu gave more variation. The highest stoichiometry obtained was 1.25 mol of phosphate/mol of β_2 , probably reflecting efficient inhibition by okadaic acid. However, the lower stoichiometry (0.62 mol of phosphate/mol of β_2) resulted from incomplete inhibition by okadaic acid, as was also observed in tryptic phosphopeptide maps of β_2 (Figure 1D). Thus a mean

Table 2 Stoichiometry of β_2 phosphorylation

^{32}P -labelled T-cells were activated with either PDBu alone (Expts. 1–3) or with PDBu after pretreatment with okadaic acid (OA) (Expts. 4–7). The specific radioactivity of ATP was determined after extraction of nucleotides; the amount of β_2 subunit was determined after immunoprecipitation of the integrin and scanning. The stoichiometry of the β_2 phosphorylation was measured by comparing the incorporation of ^{32}P from the γ -phospho group of ATP into the β_2 subunit. The mean stoichiometric values were derived from separate experiments.

Experiment	Treatment	Specific radioactivity (d.p.m./pmol)		Stoichiometry of β_2 phosphorylation	
		ATP	β_2	Values	Mean $\pm$ S.D.
1	PDBu	170.2	10.9	0.13	0.12 $\pm$ 0.01 (Expts. 1–3)
2	PDBu	122.4	6.6	0.11	
3	PDBu	122.4	6.9	0.11	
4	OA + PDBu	276.4	85.0	0.62	
5	OA + PDBu	86.1	53.9	1.25	0.92 $\pm$ 0.32 (Expts. 4–7)
6	OA + PDBu	211.5	69.2	0.65	
7	OA + PDBu	120.4	69.3	1.15	

stoichiometry of 0.92 mol of phosphate/mol of β_2 subunit was obtained with a relatively high standard deviation.

DISCUSSION

The affinity and avidity of integrins for their ligands is often regulated in response to cytoplasmic signals generated by the stimulation of cellular receptors [2–5]. One possible mechanism underlying this ‘inside-out’ activation of integrins has been hypothesized to be the phosphorylation of integrin β subunits. Different integrins have been shown to become phosphorylated on tyrosine residues [16–19] but because the tyrosine phosphorylation events are generally induced by ligand engagement, it is believed to mediate mainly ‘outside-in’ signal transduction by integrins. However, some integrins, namely β_2 [28–31] and β_3 [23], have been phosphorylated as a result of cytoplasmic signals after cellular activation. Phosphorylation of β_3 is believed to be involved in the affinity modulation of the platelet integrin $\alpha_{\text{IIb}}\beta_3$ [42].

The phosphorylation state of β_2 leucocyte integrins has been reported to change in leucocytes activated by phorbol esters [28–31]. The α chain is constitutively phosphorylated in leucocytes, whereas the β subunit becomes phosphorylated only after activation. The major phosphorylation site has been identified as Ser⁷⁵⁶ in the β_2 cytoplasmic domain [15] but the phosphorylation in this serine residue is believed not to be involved in the activation of β_2 integrin, because Ser⁷⁵⁶-mutated integrin is still activated by phorbol esters [15]. In the same study the mutation of the putative threonine phosphorylation sites (758–760) in β_2 caused a marked decrease in the adhesive function of leucocyte integrins; however, almost no phosphorylation was detected in threonine residues. A strong threonine phosphorylation of the β_2 subunit was then found by using the serine/threonine phosphatase inhibitor okadaic acid [32]. This dynamic threonine phosphorylation of the β_2 subunit was observed in T-cells activated either by phorbol esters or by antibodies against the TCR complex but not in T-cells treated with okadaic acid alone, as has been reported to occur in neutrophils [43].

This study was designed to characterize further the phosphorylation events occurring in the β_2 integrins in T-cells after activation by phorbol ester. It was first of interest to identify the amino acids transiently phosphorylated in β_2 . Tryptic phosphopeptide mappings of β_2 integrins were performed from ^{32}P -labelled T-cells activated with either PDBu alone or with

PDBu after preincubation with okadaic acid. Activation by phorbol ester revealed a single phosphorylation site in Ser⁷⁵⁶, as observed earlier [15]. When the serine/threonine phosphatases were inhibited by okadaic acid before the activation, new phosphorylation sites were observed. Two of the threonine residues present in the β_2 cytoplasmic threonine triplet (Thr⁷⁵⁸-Thr⁷⁵⁹-Thr⁷⁶⁰) were observed to become phosphorylated. Despite much effort it proved impossible to obtain a more specific identification of the threonine residues phosphorylated; this must await more sensitive analytical methods.

Earlier reports on the functional importance of the cytoplasmic threonine residues 758–760 of β_2 in the activation of the integrins [15] makes our finding of threonine phosphorylation in this triplet interesting. We found that phosphorylation takes place in two of the three threonine residues. Earlier it was shown that mutations in all three threonine residues were needed for a maximal decrease in adhesion. When threonine residues were mutated one by one, the inhibition of activation was only partial.

When the amino acid sequences in the different integrin β subunit cytoplasmic domains involved in the phosphorylation of β_2 integrin are compared, striking similarities are observed. The β_1 subunit has a serine residue corresponding to Ser⁷⁵⁶ in β_2 as well as two threonine residues in the position where β_2 has three. The β_3 subunit lacks the serine residue in the position of Ser⁷⁵⁶ but has a Thr-Ser-Thr sequence corresponding to the threonine triplet in β_2 . Ser⁷⁵² in the sequence of β_3 is important, because the point mutation Ser⁷⁵² → Pro results in Glanzmann’s thrombasthenia [44]. However, the effect of the substitution of proline for the serine residue is likely to cause a conformational change in β_3 rather than to affect the phosphorylation of the residue [45]. In fact, the β_3 integrin was found to be phosphorylated mainly on threonine [23] and the phosphorylated threonine residue is assumed to be the C-terminal residue in the β_3 cytoplasmic tail [42]. The β_7 subunit shows the closest resemblance to β_2 integrin phosphorylation sites, having both a single serine residue in the position of Ser⁷⁵⁶ as well as a threonine triplet. The only difference is that the β_2 subunit has only one residue (alanine) between the serine and threonines, whereas β_7 has two residues (alanine and isoleucine). The similarity between the β_2 and β_7 sequences in this region is intriguing, because $\alpha_4\beta_7$, like β_2 integrins, is expressed only on leucocytes and the existence of leucocyte-specific signal-transduction elements are hypothesized to be involved in the activation of both β_2 and β_7 integrins [46]. A dynamic threonine phosphorylation might also be involved in β_7 activation.

Even though the phosphorylation of β_2 integrins has been studied intensively, nothing has been known about the stoichiometry of the phosphorylation. Here we report that the phosphorylation of the β_2 subunit occurred in approx. 12% of the integrin molecules when T-cells were activated with phorbol esters; the phosphorylation took place at a single serine residue. When the phorbol ester-activated T-cells were preincubated with okadaic acid, the stoichiometry of the phosphorylation increased to 0.92 mol of phosphate/mol of β_2 subunit. This phosphorylation occurred at three different sites, suggesting that the phosphorylation takes place in approx. 30% of the integrin molecules. However, because the dynamic phosphorylation events observed are highly affected by the incomplete inhibition of phosphatases by okadaic acid, the phosphorylation might occur at one of the phosphorylation sites in β_2 more efficiently than in others.

Mechanisms by which an induced β_2 phosphorylation could regulate leucocyte adhesion are divided into two main possibilities: those resulting in an affinity change of individual integrin molecules, and those leading to an increased avidity of adhesion contacts through integrin clustering [47]. An affinity change of β_2 integrins has been reported in phorbol ester-activated T-cells in a small fraction of β_2 molecules [48], which correlates well with the relatively low stoichiometry of β_2 phosphorylation. However, recent studies indicate that neither phorbol esters nor TCR triggering could induce the high-affinity state of leucocyte integrins [49]. Rather, phorbol esters would increase the avidity of cellular contacts, probably through β_2 integrin clustering involving alterations in the cytoskeletal associations of the integrin complexes. It has previously been shown that dephosphorylation is important in β_2 -integrin-mediated adhesion [50]. We consider that the phosphorylation/dephosphorylation reactions enable the integrins to 'walk' along the submembrane skeleton, thus forming integrin clusters and resulting in increased avidity. An involvement of β_2 -integrin phosphorylation in the cytoskeletal association of the integrin complex has recently been postulated (L. Valmu, S. Fagerholm, H. Suila and C. G. Gahmberg, unpublished work).

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