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Henri Martens[a]
Onafar Kecha[a]
Chantal Charlet-Renard[a]
Marie-Paule Defresne[b]
Vincent Geenen[a]

[a] Laboratory of Radio-Immunology and Neuroendocrine-Immunology,
[b] Laboratory of Pathological Anatomy and Division of Histology, Institute of Pathology CHU-B23, University of Liège, Liège-Sart Tilman, Belgium

Neurohypophysial Peptides Stimulate the Phosphorylation of Pre-T Cell Focal Adhesion Kinases

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Abstract
Thymic oxytocin (OT) behaves as a cryptocrine signal targeted at the outer surface of thymic epithelial cell plasma membrane from where OT is able to interact with neurohypophysial peptide receptors expressed by pre-T cells. Immature T cells bear a receptor of the V_1 subtype, while OT receptors are predominantly expressed by cytotoxic CD8+ lymphocytes. In both T cell types, neurohypophysial peptide receptors transduce OT via the phosphoinositide pathway. Protein tyrosine phosphorylation is an early event of T cell activation. Western blots of murine pre-T cells (RL_{12}-NP line) proteins probed with anti-phosphotyrosine (PY-20) revealed a great number of proteins the phosphorylation of which increased either with OT or vasopressin treatment. Two were immunoprecipitated with anti-focal adhesion kinase (FAK) mAb 2A7 and were identified one as $p125^{FAK}$ and the other as a coprecipitating 130-kDa protein. The $p125^{FAK}$ is connected to the Ras/MAPK pathway and is also implicated in TCR/CD3 signalling in T cell. Another protein phosphorylated by OT in RL_{12}-NP was identified as paxillin, a 68-kDa protein localised at focal adhesion sites and associated with $p125^{FAK}$. These results indicate that phosphorylation of focal adhesion kinase may be induced in pre-T cell by thymic OT.

Introduction

The $p125^{FAK}$ is a member of a new family of non-receptor protein tyrosine kinases (PTK) which is implicated in focal adhesion, as well as in morphogenic and proliferative processes [1]. Unlike other non-receptor PTK, it lacks a structure to bind either to membranes or other cellular proteins, the *src* homology-2 (SH-2) or SH-3 domains as well as a consensus acylation site [2]. The COOH-terminal region has been identified to be critical for efficient localisation to focal adhesion sites [3]. Molecular analysis has shown that the COOH-terminal region contains a sequence called focal adhesion targeting which is able to bind to $p130^{Cas}$ and paxillin, and therefore acts through adaptator proteins Grb2/SoS to activate Ras/MAPK pathway. This PTK is stimulated in several condi-

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Henri Martens
Institute of Pathology, CHU-B23
Laboratory of Radio-Immunology, Neuroendocrine-Immunology Unit
B–4000 Liège-Sart Tilman (Belgium)
Tel. +32 43 66 25 37, Fax +32 43 66 29 77, E-Mail hmartens@ulg.ac.be

tions including oncogenic transformation and stimulation with integrins and mitogenic peptides such as bombesin, endothelin or vasopressin (VP) [4]. The $p125^{FAK}$ is ubiquitous and has been immunodetected in human T and B cells [5].

The thymus is the primary organ of T cell development and close interactions between differentiating T lymphocytes and thymic epithelial cells (TEC) play an important role in the establishment of positive/negative selection of T cells [6]. The thymus is a privileged site where the neuroendocrine system may modulate T cell differentiation, and where the immune system is educated to recognize different neuroendocrine polypeptide families [7, 8]. TEC are able to present neuroendocrine self-antigens via the major histocompatibility complex machinery [9, 10]. This process is thought to induce the central deletion of self-reactive T cells oriented against neuroendocrine families [8]. On the other hand, TEC send signals to specific receptors on pre-T cells through a pathway described as cryptocrine signalling [11].

TEC from different species synthesise neurohypophysial peptides with a marked predominance for oxytocin (OT) [12, 13]. Immature and cytotoxic T cell lines bear specific receptors for the neurohypophysial peptide family and these receptors are able to transduce neurohypophysial signals through the phosphoinositide pathway [14]. The KD of the receptors is in good accordance with the concentration of immunoreactive (IR) OT measured in the human thymus. In addition, the incorporation of tritiated thymidine is enhanced in freshly isolated human thymocytes treated with neurohypophysial-related peptides in serum-free medium [14].

An ultrastructural study has shown the diffuse presence of IR OT in the cytosol of TEC in close contact with pre-T cells and various points of focal adhesion were evidenced between the plasma membranes of TEC and pre-T cells [15]. Since $p125^{FAK}$ was shown to be implicated in T cell adhesion [16], we have investigated the hypothesis that neurohypophysial peptides may be implicated in the formation of focal adhesion sites between TEC and pre-T cells.

Materials and Methods

Cell Culture

RL12-NP cells are an immature T cell line derived from a murine radio-induced thymic lymphoma [17]. Neither CD4 nor CD8 have been found on these cells. They were maintained and propagated for experimental purposes as previously described [14]. Culture medium was RPMI 1640 supplemented with 10% FCS (Gibco BRL, Belgium, Batch 40Q1052J), 2 m*M* *L*-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, cocktail of non-essential amino acids (Bio-Whittaker, Belgium) and 10 µ*M* 2-β-mercaptoethanol (Gibco BRL, Belgium).

Immunoprecipitations

Cells were washed 3 times with a large volume of Dulbecco's modification of phosphate-balanced salt solution without Ca^{2+} and Mg^{2+} (DPBS w/o Ca/Mg) (Bio-Whittaker, Belgium) at 4°C, 400 *g* and resuspended in experimental culture medium (culture medium without FCS and 2-β-mercaptoethanol) at 10^8 cells/ml. This suspension was diluted to 10^7 cells/ml in 1 ml experimental medium at 37°C containing appropriate concentrations of the following peptides: VP (Sigma), OT (Sigma) and an antagonist to V_1 receptor d(CH2)5[Tyr(Me)2]AVP(A) (ICN Flow) [18]. The latter has been successfully used to inhibit RL_{12}-NP cells proliferation [14]. The moment of dilution was considered to be time 0. Stimulation was achieved by addition of a 5-fold volume of DPBS w/o Ca/Mg at 4°C. Protein extraction was done essentially as described elsewhere [19]. Briefly, suspensions were centrifuged at 4°C, 400 *g* and lysed at 4°C by 1 ml of a solution 150 m*M* NaCl, 0.1% NaN_3, 50 m*M* Tris HCl, pH 7.6 containing 2 m*M* EDTA, 1 m*M* Na *o*-vanadate, 10 µg/ml leupeptin, 0.1% triton X-100 and 0.1% Na deoxycholate. The lysates were preclarified by centrifugation at 12,000 *g* for 15 min. Supernatants were concentrated to 50 µl by Centricon SR-10 microconcentrator (Amicon Grace, France). Resulting protein concentrations were measured using the Bradford method in order to normalise the following operations. Total fractions were either directly submitted to electrophoresis or immunoprecipitated with anti-$p125^{FAK}$ monoclonal antibodies (mAb) 2A7 or anti-paxillin mAb (ICN Flow, clone 349) pre-coupled to protein-A-like agarose and immunoprecipitates were washed twice with PBS before electrophoresis.

Identification of Phosphotyrosine-Containing Proteins by Immunoblotting

Cell lysates or immunoprecipitates were boiled for 3 min in SDS 2% sample buffer and submitted to SDS-PAGE on 7.5% gels using the Schägger and von Jagow [20] procedures. Proteins were transferred to Immobilon (Millipore, Belgium) and the blot was incubated 12 h at 4°C in NaCl 150 mmol/l, Tris-HCl 50 mmol/l, pH 7.5 (TBS) containing 1% (w/v) blocking reagent (Boehringer Mannheim blocking reagent, Boehringer Mannheim). The blot was probed with anti-phosphotyrosine mAb PY-20 (ICN Flow, Belgium) at 1/2,000 dilution for 12 h at 4°C. Immobilon membrane was washed twice in TBS containing 0.1% Tween and twice in TBS containing 0.5% (w/v) blocking reagent for 10 min each. Incubation was then performed with 1/2,000 dilution of horseradish peroxidase (HRP)-conjugated sheep anti-mouse IgG (Boehringer Mannheim) in TBS with 0.5% (w/v) blocking reagent. Immunoreactivity was determined using the BM chemiluminescence assay reaction (Boehringer Mannheim) called thereafter fluorography. In some cases, anti-phosphotyrosine mAb was stripped of membranes after analysis by 1/hour treatment at 60°C with TBS solution containing 2% Tween-20 and 0.1 *M* β-mercaptoethanol. The efficiency of the stripping was confirmed by incubating blots in developing reagents and re-exposing to autoradiography film. Finally, the stripped membrane was probed with 2A7 anti-FAK in order to confirm similar loading. Quantification of phosphorylation was performed using densitometric analysis of fluorography performed with a Bio-Rad GS-700 imaging densitometer and calculated with the Molecular Analyst 1.4 Bio-Rad software. Normalisation of the intensity was made using either the resid-

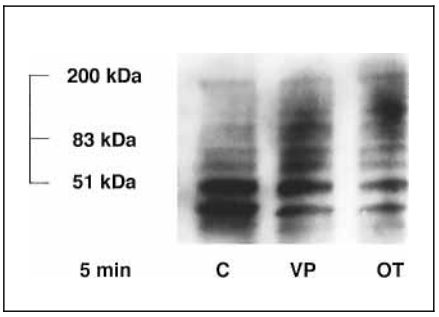

Fig. 1. Induction of phosphorylation in VP- and OT-treated cells. 10^7 RL_{12}-NP cells were incubated for 5 min in absence (C) or in presence of VP (1 n*M*) or OT (1 n*M*) before addition of cold DPBS w/o Ca/Mg. Lysates were submitted to electrophoresis and transferred on Immobilon membranes. Membranes were probed with PY-20 mAb specific for phosphotyrosine residues. IR proteins were visualised by fluorography. The basal level of phosphorylation might indicate a constitutive activation of intracellular proteins related to the lymphomatous nature of the cell line.

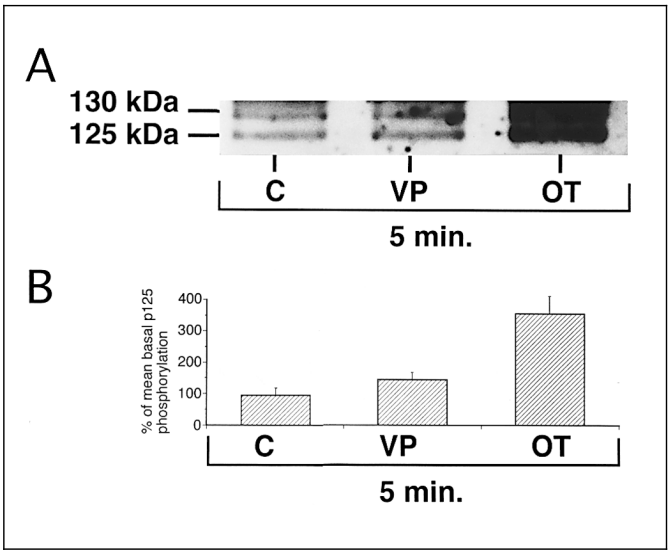

Fig. 2. Comparison between OT- and VP-induced phosphorylation. 10^7 RL_{12}-NP cells were incubated in non-supplemented (C), OT (1 n*M*) or VP (1 n*M*) supplemented medium for 5 min. **A** Lysates were submitted to immunoprecipitations with mAb 2A7 protein A-like agarose conjugated before electrophoresis. Proteins transferred on Immobilon were probed with PY-20 mAb specific for phosphotyrosine residues. **B** Quantification of $p125^{FAK}$ phosphorylation. Fluorography from 3 separate experiments were analysed and expressed as mean ± SD of percentage of basal $p125^{FAK}$ phosphorylation.

ual IgG band that appeared on the membrane by reaction with the HRP-conjugated anti-IgG or the reprobing of the membrane with anti-FAK antibody. Both of the normalisations gave similar results.

Statistical Analyses

At each time point, values obtained from multiple experiments were reported as percentage of mean basal phosphorylation value. Compiled data from at least four separate experiments were compared using the ANOVA test. Statistical significance was accepted for $p \leq 0.05$.

Results

Effect of Neurohypophysial Peptides on the Phosphorylation of RL_{12}-NP Proteins

To determine whether neurohypophysial-related peptides increase protein phosphorylation in RL_{12}-NP cells, proteins were extracted from cells which had been incubated with or without OT or VP at 1 n*M* for the indicated time. Those peptides were appropriate candidates, according to the specificity of the neurohypophysial receptor expressed by those cells [14, 21] or by other T cell lines [22, 23]. Five minutes after addition of either OT or VP, the cells were lysed as described in experimental procedures and lysates were analysed on Western blot with the anti-phosphotyrosine PY-20 mAb.

This result is similar to the VP-induced tyrosine phosphorylation in fibroblasts [24] known to bear a V_1-type receptor [25]. VP and OT treatments modulate phosphorylation of a wide range of proteins in the pre-T cell lines as shown in figure 1. The rate of phosphorylation was increased by OT and VP in the protein fractions between 100 and 200 kD. This rate was reduced after VP and even reached a lower level after OT in fractions below 51 kD. Nevertheless, the precise identity of phosphorylated proteins could not be deduced from their apparent molecular weight.

Lysates of 5-min untreated or treated cells were immunoprecipitated with anti-$p125^{FAK}$ 2A7 mAb. Western blotting of immunoprecipitates analysed with PY-20 anti-phosphotyrosine mAb is shown in figure 2. There was a marked increase of the phosphorylation rate in two bands with an apparent molecular weight of 125 and 130 kD when RL_{12}-NP cells were treated with OT for 5 min. An increase was also observed in VP-treated RL_{12}-NP cells (fig. 2A). Densitometric analysis revealed that OT-induced $p125^{FAK}$ phosphorylation twice as high as VP (fig. 2B). Statistical analyses confirmed a significant difference between VP- and OT-induced $p125^{FAK}$ phosphorylation (VP vs. control C; $p < 0.05$, OT vs. C: $p < 0.01$, OT vs. VP: $p < 0.05$ using ANOVA test).

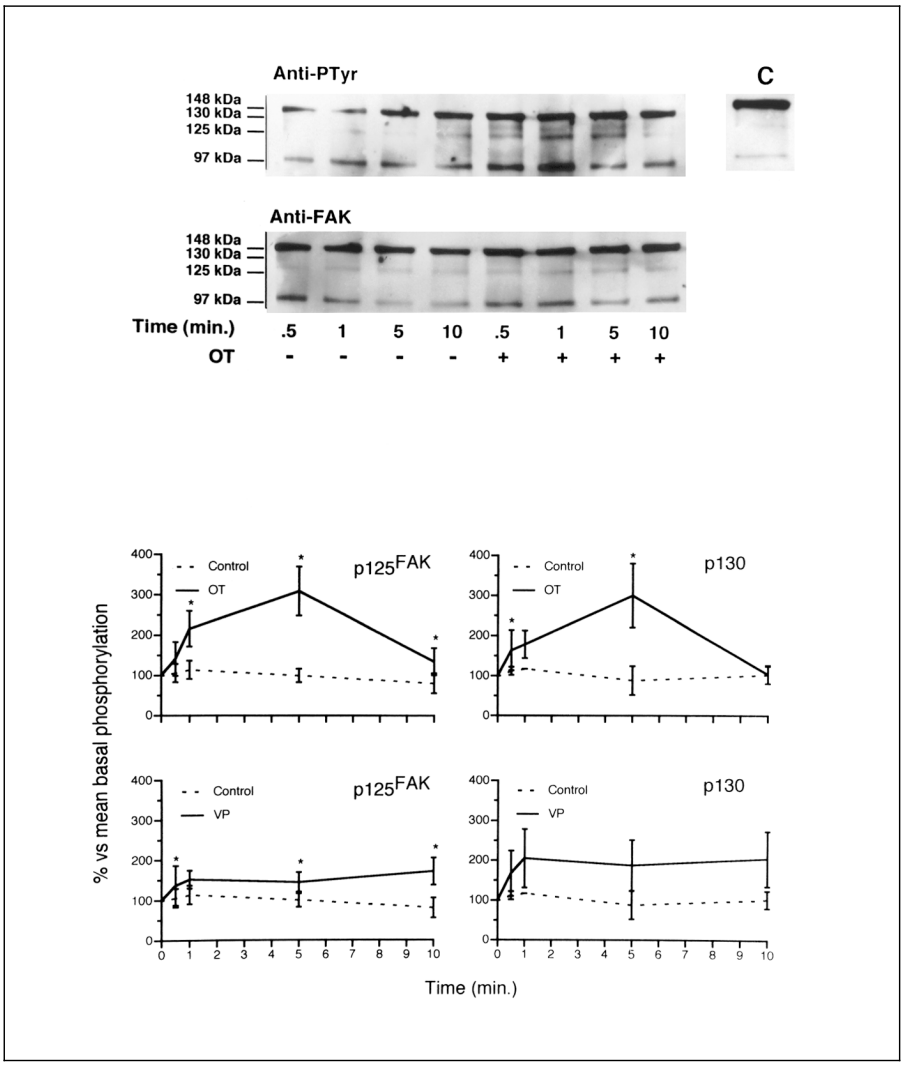

Fig. 3. Upper panel: Time course of phosphorylation in anti-p125^{FAK} immunoprecipitates of proteins extracted from OT-stimulated RL_{12}-NP cells. RL_{12}-NP cells were stimulated during various times in absence (–) or in presence (+) of OT (1 n*M*) before addition of cold DPBS w/o Ca/Mg. Lysates were submitted to immunoprecipitations with mAb 2A7 protein A-like agarose conjugated before electrophoresis. Anti-PTyr: proteins transferred on Immobilon were probed with PY-20 mAb specific for phosphotyrosine residues. Anti-FAK: the same membrane was stripped and blotted with 2A7 mAb to demonstrate similar loading. Lane C is control mAb 2A7 protein A-like agarose conjugated probed with anti-IgG second antibody alone. IR proteins were visualised by fluorography. Lower panel: Analysis of the time course of phosphorylation induced by OT and VP in RL_{12}-NP cells. Each point represents the mean and vertical bars represent the SD of at least four separate experiments. Upper left corner: relative intensity of p125^{FAK} phosphorylation induced by OT. Lower left corner: relative intensity of p125^{FAK} phosphorylation induced by VP. Upper right corner: relative intensity of p130 phosphorylation induced by OT. Upper left corner: relative intensity of p130 phosphorylation induced by VP. * $p < 0.05$: OT- or VP-induced phosphorylation vs. basal phosphorylation at each time (ANOVA test).

Time Course of Tyrosine Phosphorylation of p125^{FAK} in RL_{12}-NP Cells

RL_{12}-NP cells were then incubated with OT or VP (1 n*M*) during the indicated time, immunoprecipitations with 2A7 mAb protein A-like agarose-conjugated and a two-step revelation was performed according to the procedure described (fig. 3).

The phosphorylation rate was markedly increased in two bands located at 125 kDa (assumed to be p125^{FAK}) and 130 kDa. The 97 kD Ig band represents a single dimer of heavy Ig chains [26]. This is shown in figure 3 (lane C) where 2A7 mAb linked to a protein-A-like agarose was probed with HRP-conjugated anti-IgG alone. As precipitates were eluted by boiling for 3 min in SDS sample buffer, a 148-kDa IgG_1 band appeared as 2A7 mAb released from protein-A-like agarose as shown by the same band in control immunoprecipitates in figure 3 (lane C). To demonstrate a similar loading of the immunoprecipitated proteins, anti-phosphotyrosine mAb was stripped as described in 'Material and Methods' and blotted with 2A7 mAb in the same two-step procedure. Only the 125-kD band appeared on this blot (fig. 3, anti-FAK) with similar intensities in each conditions. Similar features were obtained when VP was used instead of OT.

The fluorography was quantified by a Bio-Rad GS-700 imaging densitometer and Molecular Analyst 1.4 Bio-Rad program in order to estimate the time course evolution of phosphorylation induced by OT or VP (fig. 3, lower panel). From 1 to 10 min in basal conditions, there was no significant variation in the phosphorylation rate of p125^{FAK}. With OT at 1 n*M*, there was a time-dependent phosphorylation of the p125^{FAK} and of the 130-kDa pro-

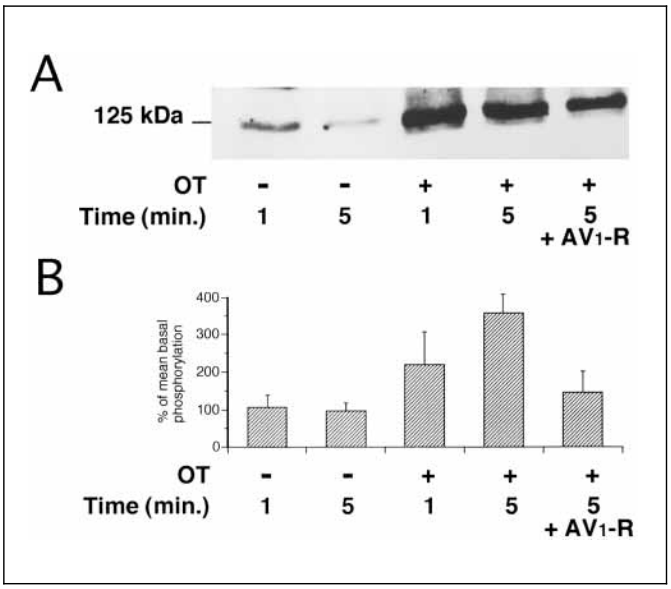

Fig. 4. Inhibition of OT-induced phosphorylation by a V_1 antagonist. 10^7 RL_{12}-NP cells were incubated in non-supplemented (–), OT (1 n*M*)-supplemented medium (+) for 1 or 5 min, or with OT (1 n*M*) and V_1 antagonist 10 n*M* for 5 min (+AV_1-R). **A** Lysates were submitted to immunoprecipitations with mAb 2A7 protein-A-like agarose conjugated before electrophoresis. Proteins transferred on Immobilon were probed with PY-20 mAb. **B** Quantification of $p125^{FAK}$ phosphorylation. Fluorography from 3 separate experiments were analysed and expressed as mean ± SD of percentage of basal $p125^{FAK}$ phosphorylation.

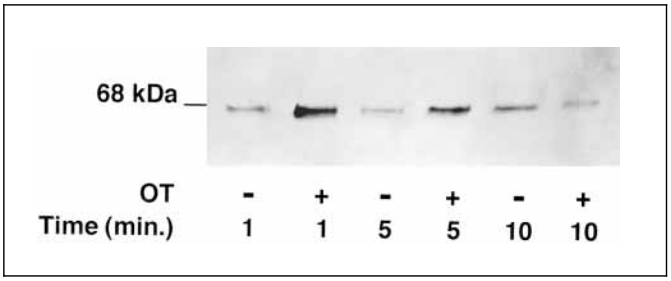

Fig. 5. Paxillin phosphorylation under OT stimulation. 10^7 RL_{12}-NP cells were incubated in non-supplemented (–), or OT (1 n*M*)-supplemented medium (+) for 1, 5 or 10 min. Lysates were submitted to immunoprecipitations with anti-paxillin mAb 349 (ICN Flow) protein A-like agarose conjugated before electrophoresis. Proteins transferred on Immobilon were probed with PY-20 mAb specific for phosphotyrosine residues.

tein (upper graphs) leading to more than 3-fold in the OT-stimulated band versus basal conditions. VP also induced the phosphorylation of both $p125^{FAK}$ and the 130-kDa protein (lower graphs) which reached a double intensity compared to the basal level after 5 min. VP-induced phosphorylation of $p125^{FAK}$ reached a maximum after 1 min, while OT-induced phosphorylation increased until 5 min and decreased after 10 min.

Inhibition of VP- and OT-Induced Phosphorylation by a Specific V_1 Antagonist

The effects of an anti-V_1 antagonist could be evidenced in OT-stimulated RL_{12}-NP cells. This antagonist reduced the phosphorylation of $p125^{FAK}$ with a high potency, being already active at 10 n*M* (fig. 4A, B). Statistical analysis confirmed that the OT-induced phosphorylation of $p125^{FAK}$ was significantly reduced by addition of the V_1 antagonist (OT at 5 min vs. basal at 5 min: $p < 0.01$; OT at 5 min vs. OT + V_1 antagonist at 5 min: $p < 0.05$ using ANOVA test). The phosphorylation of $p125^{FAK}$ induced by VP was also inhibited by a V_1 antagonist (data not shown).

Effect of Neurohypophysial Peptides on Paxillin Phosphorylation

When an anti-paxillin instead of 2A7 mAb was used to immunoprecipitate RL_{12}-NP lysates, the stimulation of paxillin by neurohypophysial peptides could be evidenced in RL_{12}-NP cells.

Figure 5 shows that paxillin phosphorylation is increased by OT with a time course similar to $p125^{FAK}$ phosphorylation. However, the paxillin phosphorylation decreased under the basal level at 10 min. VP also induced paxillin phosphorylation with a similar time course (data not shown).

Discussion

This study shows that $p125^{FAK}$ proteins are present in RL_{12}-NP pre-T cell line, and that neurohypophysial peptides are able to stimulate their phosphorylation in a time-dependent manner with a maximum occurring within 1–5 min. These results concord with previous observations [27, 28] which showed that VP stimulates $p125^{FAK}$ and paxillin phosphorylation in a fibroblast cell line. However, OT appears to be more potent than VP to induce phosphorylation in RL_{12}-NP pre-T cell line.

The rapidity of neuropeptide-induced phosphorylation is consistent with mechanisms of early activation and indicates that $p125^{FAK}$ may function as a downstream ele-

ment in a neuropeptide-stimulated tyrosine kinase pathway. The 130-kDa protein detected in anti-p125^{FAK} immunoprecipitates the phosphorylation of which increased together with p125^{FAK}, could represent a coprecipitating substrate of p125^{FAK}, a coprecipitating protein kinase or a protein that non-specifically bind to the immune complexes. It is interesting to note that, like the p125^{FAK}, there is a protein of 130 kDa called p130^{Cas}, which is a major tyrosine phosphorylated protein in *src*-transformed fibroblasts which intervenes in adhesion mechanisms, and which forms stable complexes with activated forms of pp60$^{v\text{-}src}$ [29–33]. The p130^{Cas} may be phosphorylated by VP in a fibroblastic model [27]; it may associate both in vitro and in vivo with p125^{FAK}, and it may function as a mediator in transduction of adhesion signals [34, 35]. The phosphorylation of the p125^{FAK}-associated proteins p130 (probably p130^{Cas}) and paxillin was also evidenced in RL_{12}-NP cells. It has been suggested that p130^{Cas} and paxillin phosphorylations are regulated by p125^{FAK} because these proteins colocalize in cellular focal adhesions, are physically associated with p125^{FAK} and are coordinately phosphorylated on tyrosine residues in response to multiple stimuli [1]. This study shows that neurohypophysial peptides induce the phosphorylation of at least three focal adhesion-associated proteins in RL_{12}-NP pre-T cells.

RL_{12}-NP is a pre-T cell line derived from a thymic lymphoma induced by X-ray irradiation of C57BL mice. RL_{12}-NP cells are characterised by the absence of CD4 and CD8 differentiation markers (like double-negative thymocytes), and by the presence of functional neurohypophysial receptors with characteristics close to the V_1 subtype expressed in the thymus [36, 37]. Thus, a series of phenotypical and functional aspects are concordant between thymocytes and RL_{12}-NP cells. Since freshly isolated thymocytes are highly heterogeneous, there is an advantage to use of homogenous cell line. In addition, the inhibition of p125^{FAK} phosphorylation in RL_{12}-NP cells by an antagonist directed against a V_1 receptor confirms our previous study which showed that pre-T cells bear a V_1 receptor [14].

The precise nature of the thymic natural ligand interacting with V_1 receptors on pre-T cells may be questioned. The predominant neurohypophysial peptide expressed in the thymus is OT and only mAbs to OT are able to activate cytokine secretion by TEC [38]. As shown in this study, OT is more potent than VP to induce p125^{FAK} phosphorylation and this OT effect is inactivated by a V_1 antagonist. These findings suggest that OT could be a natural ligand of the V_1 receptors borne by thymic T cells. It should be noticed that OT has already been shown to act through V_1-type receptors in other systems [39, 40].

The p125^{FAK} has been shown to be a potential activator of the adaptator protein system Grb2/SoS leading to stimulation of the ras/MAPK pathway [41] and also to act through the PI 3-kinase pathway [42, 43]. Therefore, the mitogenic effect of OT and VP observed on human and murine freshly isolated thymocytes could be mediated by activation of p125^{FAK}.

The implication of p125^{FAK} in thymic T-cell development and activation is suggested by several experimental facts such as the presence of p125^{FAK} mRNA in human T and B cells [5], as well as in murine thymocytes at different differentiation stages [44]. A p125^{FAK}-related protein (called FakB) has been shown to be phosphorylated following TcR activation [45] and p125^{FAK} itself is synergistically stimulated in CD4+ cell blasts and Jurkat T cell line by integrins and TcR engagement [46, 47]. With regard to the kinetic of p125^{FAK} phosphorylation, the transient maximum observed with OT has also been shown in T cells stimulated by chemokine RANTES [16], while TCR engagement or integrin activation induced a more sustained phosphorylation of p125^{FAK} [46–49]. Furthermore, while it is well established that apoptosis is crucial in the process of thymic T cell selection [50], a relationship has been shown in human tumor cells between the attenuation of expression of p125^{FAK} and the induction of apoptosis [51]. Finally, focal adhesion proteins play a fundamental role in early embryogenesis as evidenced by the defect in mesoderm induction in p125^{FAK} knockout mice [52, 53]. The observations of numerous points of focal adhesion using electron microscopy constituted a preliminary hypothesis for the investigation of p125^{FAK} implication in T cell development. Our results confirm this hypothesis and provide a biochemical link with the ultrastructural data [15].

p125^{FAK} has been discovered in a model of *v-src*-transformed chicken embryo [2] and was shown to be an activator of pp60^{src} [54]. Moreover, increased p125^{FAK} expression is associated with invasive and metastatic tumour phenotype [55–59]. Some p125^{FAK} antisenses have been proposed as pharmacological agents against cancer [60]. Thus, the basal and stimulated phosphorylation of p125^{FAK} in a T cell line derived from a radioinduced lymphoma could be correlated with the immortalised phenotype. The inhibitory effect of a V_1 receptor antagonist on p125^{FAK} phosphorylation induced by neurohypophysial peptides also suggests that such a pharmacological agent could be tested for its putative antiproliferative and antimetastatic actions.

The stimulation of pre-T cell kinases implicated in adhesion and proliferation by signals encountered within thymic microenvironment further supports the existence of a cryptocrine signalling effective in vivo between TEC and developing T lymphocytes [8, 11, 38]. Obviously, the phosphorylation of focal adhesion kinases induced by natural thymic peptide ligands deserves to be further investigated as an important pathway of T cell differentiation and selection.

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