Phosphorylation of Extracellular Domains of T-Lymphocyte Surface Proteins

CONSTITUTIVE SERINE AND THREONINE PHOSPHORYLATION OF THE T CELL ANTIGEN RECEPTOR ECTODOMAINS*

Sergey G. Apasov, Patrick T. Smith, Marie T. Jelonek, David H. Margulies, and Michail V. Sitkovsky†‡

From the Laboratory of Immunology, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20892-1892

The extracellular accumulation of ATP after activation of T-lymphocytes, as well as the presence of ecto-protein kinases in these cells, led us to propose that T cell surface receptors could be regulated through the reversible phosphorylation of their extracellular domains (ectodomains). Here, in a model system, we used T cell transfectants which express T cell antigen receptor chains lacking intracellular and transmembrane protein domains and 32P, metabolic labeling of cells to definitively demonstrate phosphorylation of ectodomains of T cell surface proteins. We show that αβ TCR ectodomains were phosphorylated intracellularly and constitutively on serine and threonine residues and were then expressed on the T cell surface in phosphorylated form. TCR ectodomains also could be phosphorylated at the cell surface when extracellular [γ-32P]ATP or [γ-32P]GTP were used as phosphate donors with the same cells. Consensus phosphorylation sites for serine and threonine protein kinases were found to be strongly evolutionary conserved in both α and β TCR chains constant regions. These results are consistent with the hypothesis, where T cell surface proteins which are phosphorylated intracellularly on their ectodomains, could subsequently be expressed at the cell surface and then be reversibly modified by ectoprotein phosphatase(s) and by ectokinase(s). Such modifications may change T cell cognate interactions by, e.g. affecting TCR-multimolecular complex formation and antigen binding affinity. It is suggested that αβ TCR ectodomain phosphorylation could serve as a potential mechanism for regulation of αβ TCR-mediated T-lymphocytes response.

In addition, extracellular domains of cell surface proteins are involved in multimolecular complex formation as underscored by studies of TCR/CD3 subunit assembly (3) and of CD4-TCR interactions that contribute to TCR recognition of MHC class II-presented peptides (4). It was also demonstrated recently that CD45 ectodomains regulate CD4-αβ TCR associations (5).

These considerations and our earlier studies of the role of extracellular ATP in T cell effector functions (6) led us to propose that properties of T cell surface receptors, including cell adhesion proteins and recognition molecules, could be regulated through phosphorylation of their extracellular domains (7, 8) in a manner now accepted as a mechanism for regulation of enzyme-substrate interactions (9).

The precedent for such a mechanism was recently provided by studies of CD36 on the platelets surface. It was demonstrated that the specificity of that receptor for collagen or thrombospondin is regulated by ectophosphorylation of its ectodomain (10).

Analysis of published amino acid sequences of lymphocyte surface proteins (data not shown) revealed the presence of consensus protein kinase phosphorylation sites in the extracellular domains of the majority of functionally important surface proteins, including αβ TCR. αβ TCR was also found to be ectophosphorylated by extracellular [γ-32P]ATP during preliminary screening of immunoprecipitates of T cell surface proteins with a panel of monoclonal antibodies (8).2 This suggested that αβ TCR ectodomain phosphorylation could affect antigen recognition and/or interactions of αβ TCR molecules with ectodomains of other functionally important molecules (e.g. CD4/CD8, CD3, or CD45) and thereby regulate T cell cognate interactions and effector functions (8).

The unambiguous demonstration of T cell surface proteins ectodomain phosphorylation is an important requirement for the further testing of this hypothesis since the studies of extracellular protein kinases (ectokinases) in other cellular systems (11, 12) were not complemented by definitive description of their cell surface substrates due to known caveats of ectophosphorylation assays using extracellular [γ-32P]ATP (13).

In this study we provide the first direct evidence of the T cell surface protein (αβ TCR) ectodomain phosphorylation. This was done by using T cell transfectants that express the recombinant intracellular tail and transmembrane domain-lacking αβ TCR molecules after the long-term metabolic 32P, labeling.

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† To whom correspondence and reprint requests should be addressed. The abbreviations used are: MHC, major histocompatibility complex; αβ TCR, αβ T cell receptor; CKII, casein II kinase; GPI, glycoporphatidylinositol; mAb, monoclonal antibody; PLC, phospholipase C; 32P, inorganic phosphate; PAGE, polyacrylamide gel electrophoresis.

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Flow Cytometry Analysis—Flow cytometry analysis was performed on FACScan using the CellQuest program after standard cell staining with phosphate-buffered saline and rabbit anti-mouse FITC-labeled antibody (Sigma). The monoclonal antibody reacted with the anti-TCR (H57–597) and was used for the analysis of T-cell activation. The samples were acquired using a flow cytometer (EPICS C, Coulter Electronics) and analyzed using CellQuest software (Becton Dickinson). The data were expressed as mean fluorescence intensity (MFI) for each sample.

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peptide, as detected by plasmon resonance assay (Fig. 1C). The soluble 2C-TCR was also found to bind to clonotypic 1B.2 mAb in a parallel experiment (data not shown). Taken together, these results justified the use of a procedure involving PLC pretreatment of 2C-TCR transfectants followed by immunoprecipitation with mAb to TCR for the specific purification of 2C-αβTCR molecules in order to study their ectodomain phosphorylation.

Detection of αβTCR Ectodomain Phosphorylation—After 4 h of metabolic labeling with 32P, followed by cell lysis and immunoprecipitation with the clonotypic anti-2C-TCR mAb we observed phosphorylated TCR molecules on SDS-PAGE gel. 32P-Labeled 2C-TCR is seen as disulfide linked heterodimer that comigrates with 125I-surface-labeled TCR (Fig. 2A).

Phosphorylated 2C-TCR molecules were released from the cell surface by PLC treatment after 16 h of metabolic cell labeling and were detected in the supernatants by immunoprecipitation with clonotypic 1B.2 mAb and SDS-PAGE under reducing and non-reducing conditions similar to 125I-surface-labeled TCR (Fig. 2; panels C and D, lanes 4). In control samples no αβTCR bands were observed when cells were not exposed to PLC (Fig. 2; C and D, lanes 3 and 5) or if no 1B2 mAb was used (lanes 1 and 2). H57–597 mAb anti-mouse TCR common epitope was used with the same results (data not shown) as clonotypic mAb 1B.2. These results are consistent with the conclusion that the 32P-labeled proteins detected in anti-TCR mAb immunoprecipitates (Fig. 2) were, indeed, the GPI-linked
alpha beta TCR chains.

The detailed time course of the cell labeling with $^{32}$P and PLC-treatment (Fig. 2B) demonstrated that $^{32}$P-labeled alpha beta TCR molecules could be detected on the cell surface as early as after 1 h and established 16 h as the best and most convenient time of $^{32}$P labeling to observe the $^{32}$P-phosphorylated and PLC-releasable alpha beta TCR molecules.

Phosphoamino acid analysis of SDS-PAGE separated TCR protein bands both under reducing (Fig. 3, panels A and B) and non-reducing conditions (Fig. 3, panels C and D) conditions revealed the presence of phosphoserine and phosphothreonine, but not phosphotyrosine. The same results were obtained by phosphoamino acid analysis of TCR isolated by immunoprecipitation from either total cell extracts of $^{32}$P metabolically labeled T cells (data not shown) or from the PLC-releasable fraction (Fig. 3).

Detection of phosphoserine and phosphothreonine also excluded the possibility that the radioactivity observed on autoradiographs (Fig. 2) in $\alpha\beta$2C-TCR chains was due only to the $^{32}$P-labeled phosphoinositol, which was released with alpha beta TCR ectodomains after treatment with PLC. It is of interest that some yet-to-be identified T-cell phosphoproteins were co-immunoprecipitated with TCR (Fig. 2) and were ectophosphorylated by $[^{32}\text{P}]\text{ATP}$ and $[^{32}\text{P}]\text{GTP}$ (Fig. 5).

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Phosphorylation of $\alpha\beta$TCR molecules after $^{32}$P labeling of 2B4 T cell cells. Evolutionary conserved protein kinases consensus phosphorylation sites. 2B4 cells were incubated with $^{32}$P, for 16 h (Panel A) or cell surface-labeled with $^{125}$I (Panel B), immunoprecipitates were obtained from total cell lysates described under "Materials and Methods" and resolved in SDS-PAGE under reducing conditions. Panel A, intracellularly $^{32}$P-phosphorylated 2B4 $\alpha\beta$TCR molecules are demonstrated after immunoprecipitation with H57–597 hamster anti-mouse TCR mAb (lane 3) but not with hamster anti-mouse CD28 mAb (lane 2) or nonspecific clonotypic anti-2C-TCR mAb 1B.2 (lane 4) or protein A beads alone (lane 1). Panel B, cell surface $^{125}$I-labeled $\alpha\beta$TCR molecules were immunoprecipitated (in the same conditions as in Panel A) from 2B4 cells with H57–597 hamster anti-mouse TCR mAb (lane 2) but not with protein A beads alone (lane 1). Positions of $\alpha\beta$TCR are indicated by arrows. Panel C, conserved protein kinase consensus phosphorylation sites found in human, rabbit, and mouse $\alpha\beta$TCR ectodomains. The published amino acid sequences of $\alpha\beta$TCR were inspected for the presence of protein kinase consensus phosphorylation sites as indicated in the figure.
The strikingly evolutionary conserved (between rabbit, human, and mouse) protein kinase phosphorylation sites were revealed in both CaTCR and CβTCR ectodomains (Fig. 4C) by analyzing their published (22, 23) amino acid sequences. Interestingly, the CKII phosphorylation site in CβTCR is located in the solvent-exposed insertion absent in C domains of Ig, as indicated in the x-ray crystallographic structure of the TCR β chain (24).

Phosphorylation of αβTCR ectodomains in normal T cells and in GPI-TCR transfectants was also studied in an ectophosphorylation assay by using extracellular [γ-32P]ATP or [γ-32P]GTP as a phosphate donor. We observed αβTCR phosphorylation by extracellular [γ-32P]ATP in thymocytes, lymph node cells (Fig. 5, panel A), and T cell lines (Fig. 5, panels B-D).

To avoid misinterpretation (13) we introduced two new and more stringent controls: (i) the requirement to confirm the extracellular [γ-32P]ATP-mediated phosphorylation of intact cells by using transfectants with transmembrane domain and cytoplasmic tail lacking TCR molecules; (ii) the requirement for [γ-32P]ATP-mediated ectophosphorylation to be affected by pretreatment of cells with membrane-impermeable protein phosphatases. These requirements are satisfied in experiments where we observed the phosphorylation of 2C-TCR in transfectants after incubation with [γ-32P]ATP (Fig. 5, panel D) similar to the phosphorylation after 32P, metabolic labeling of the cells (Fig. 2). The pretreatment of T cells with protein phosphatases dramatically improved the [γ-32P]ATP-mediated ectophosphorylation of αβTCR as was expected if the majority of αβTCR ectodomain-phosphorylation sites has been constitutively phosphorylated (Fig. 5, panel B).

Incubation of intact cells with both extracellular [γ-32P]ATP or [γ-32P]GTP resulted in phosphorylation of proteins that were immunoprecipitated with clonotypic anti-TCR mAb (Fig. 5 and data not shown). The abilities of both [γ-32P]ATP and [γ-32P]GTP (Fig. 5, lanes 2 and 4) to serve as phosphate donors in ectophosphorylation reactions are hallmarks of the casein II kinases (25). In our earlier studies of ectophosphorylation assays with specific inhibitors and enhancers of CKII,4 we found that a casein kinase II-like enzymatic activity was the major ectokinase activity in T cells.

The results of the experiments presented above strongly suggest that ectodomain phosphorylation may take place both intracellularly and extracellularly. Importantly, the intracellular phosphorylation of αβTCR is constitutive, since no additional stimuli were required to observe it in metabolically 32P-labeled T cells. The demonstrated effects of extracellularly added protein phosphatases (Fig. 5, panel B) support the possibility of the ectodomain phosphorylation being a reversible process.

DISCUSSION

We describe here the constitutive phosphorylation of T cell surface protein ectodomains using recombinant αβTCR T cell transfectants as a model system. The possibility is raised that such phosphorylation may reflect the functioning of the previously unexplored mechanism of regulation of TCR-mediated cognate T cell’s interactions. Indeed, T lymphocytes seem to possess the complete system of reversible extracellular phosphorylation/dephosphorylation as evidenced by the extracellular ATP accumulation in TCR-triggered T cells (26), by the description of T cell-associated ATPases (6), ectokinases, and protein phosphatases (8)5 and as demonstrated here by ectodomain phosphorylation of functionally important T cell surface protein (αβTCR). The function of the highly active ecto-ATPase activities on T cells (6) could be to provide an additional level of regulation of the extracellular domains phosphorylation and signaling through purinergic receptors by limiting the concentration of the extracellular ATP near the cell surface.

Extracellular domains of αβTCR have been the subject of intensive investigations, since they contain the antigen recognition and binding sites (1) and regulate antigenic peptide recognition due to their interactions with CD4 ectodomains (4) and/or other surface proteins which form the multimolecular TCR-CD3 complex. The involvement of αβTCR ectodomain-mediated interactions is also implied by considerations of the

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4 S. A. Redegeld, P. Smith, and M. Sitkovsky, unpublished observations.
5 F. Redegeld, unpublished observations.
role of αβTCR dimerization and aggregation (27–29), lattice formation (30) in transmembrane signaling in T cells. In addition, αβTCR-CD3 ectodomain interactions were shown to be important for the assembly of TCR-CD3 molecular complexes (3).

The possibility of the role of extracellular ATP in extracellular domain phosphorylation of functionally important proteins in T cell’s effector functions was first raised during earlier studies of the biochemical mechanisms of CTL-target cell interactions (7, 8, 26) and it was important to explore whether reversible αβTCR ectodomain phosphorylation could be the fine-tuning mechanism of TCR-mediated immune responses.

The ectophosphorylation in different cellular systems has been studied over the last several decades (reviewed in Refs. 20 and 31), and elaborate criteria were developed to avoid many potential problems in interpretations of ectophosphorylation assays. Nevertheless, none of these criteria were sufficient, and intracellularly-cytosolic tail-phosphorylated proteins could be still misinterpreted as “ectophosphorylated” (13). Thus, the definitive demonstration of phosphorylation of ectodomains had yet to be provided in any cellular system, and a new approach was needed to demonstrate the presence of phosphorylated amino acid residues in αβTCR ectodomains.

The demonstration of αβTCR ectodomain phosphorylation was accomplished here using T cells stably transfected with transmembrane domain- and cytoplasmic tail-lacking GPI anchor-linked molecules of 2C-αβTCR and labeled both in a [$γ^{32}P$]ATP ectophosphorylation assay and in a long-term [$γ^{32}P$] metabolic labeling assay (Figs. 1–5). This allowed us to independently confirm experiments with [$γ^{32}P$]-labeled 2C-TCR transfectants or normal T cells with results of ectophosphorylation assay obtained by incubating intact cells with [$γ^{32}P$]ATP/GTP.

The important differences between αβTCR ectodomains and intracellular domains of the TCR-CD3 complex phosphorylation were revealed here by photoamino acid analysis (Fig. 3). In contrast to well documented intracellular domain tyrosine phosphorylation (2), only the phosphoserine and phosphothreonine, but not phosphotyrosine, were detected in our studies of αβTCR ectodomains.

The interchangeable use of ATP and GTP (Fig. 5) as phosphate donors in αβTCR ectophosphorylation has important implications, since it not only implicates ectokinase with CKII-like properties in αβTCR ectodomain phosphorylation but it also eliminates the possibility of the opening of ATP4–gated nonspecific membrane pores-P2z receptors (8). These results are consistent with experiments demonstrating the effects of CKII inhibitors and enhancers on the ectophosphorylation of surface proteins on T cells (data not shown).

Taken together, the results described above and studies of the effects of pretreatment of cells with protein phosphatases and protein kinases (Fig. 5 and data not shown) are consistent with the model in which ectodomains of TCR are constitutively intracellularly serine/threonine phosphorylated and then expressed on the cell surface. The detection of both phosphoserine and phosphothreonine indicates the possibility that there are several phosphorylation sites in each of the αβ TCR chains. Subsequently, αβTCR ectodomains can be dephosphorylated extracellularly by PP1 and PP2A ectoprotein phosphatase(s) (data not shown) and further rephosphorylated by CKII-like or other ectoenzyme with extracellular ATP/GTP as a phosphate donor. The implication of ecto-CKII-PKase in αβTCR ectophosphorylation is especially intriguing in view of strikingly evolutionary conserved (between rabbit, mouse and human) consen-

sus phosphorylation sites for CKII in the Cβ domain of αβTCR and of tandem PKC and CKII phosphorylation sites in the Ca domain (Fig. 4C).

The functional role of αβTCR ectodomain phosphorylation remains to be definitively established and the most promising approach appears to be in testing the effects of mutation of the ectophosphorylation site on cognate interactions of T cells transfected with mutated αβTCR and studies of the effects of phosphorylation of soluble recombinant TCR on TCR/MHC class I-peptide interactions using plasmon resonance techniques.

The extracellular phosphorylation system described here may have important immunopharmacologic implications, since the well defined phosphorylation sites in ectodomains of functionally important surface proteins and ectoenzymes (protein kinases and protein phosphatases) would provide attractive targets for immunomodulation due to their surface location.

In conclusion, we would like to speculate that αβTCR chains have more than one phosphorylated residue in each chain and, depending on their location, they may influence associations of αβTCR chains with CD3 molecules and/or associations of αβTCR chains with CD4/CD8 co-receptors or other molecules comprising the TCR-CD3 complex. It should be also tested whether the αβTCR ectodomain phosphorylation may affect recognition and binding properties of αβTCR variable regions thereby providing an attractive mechanism of “On/Off” switch in regulation of formation and separation of cells in CTL/target cell or Th cell/antigen presenting cells conjugates.

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