The extracellular domain of CD4 receptor possesses a protein kinase activity

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Abstract The CD4 receptor of T-helper cells is an essential participant in immune response formation and HIV infection. We report here that the extracellular domains of CD4 receptor can catalyze the phosphotransferase (kinase) reaction. Incubation of rsCD4 in solution with [γ-^{32}P]ATP results in the Ca^{2+}-dependent autophosphorylation of the protein presumably at a His residue because the reaction is prevented by the diethylpyrocarbonate treatment. The rsCD4 phosphorylates milk casein or human plasma proteins as a Ser/Thr protein kinase.

Key words: CD4 receptor; Autophosphorylation; Protein kinase; T-lymphocyte

1. Introduction

CD4, a cell surface transmembrane glycoprotein of T-helper cells, participates in several cellular processes and interactions, including T cell receptor (TCR)/CD3-mediated recognition of processed antigen/MHC (major histocompatibility complex) class II complexes on the surface of B lymphocytes and other antigen presenting cells (APC). This leads to stimulation of lymphocytes and to humoral immune responses [1–3]. The interaction of CD4 accessory molecules on T cells with MHC class II antigens on B cells is also essential for T cell activation [4–6]. Direct binding of the CD4 receptor with an invariant domain of the MHC class II molecules has been shown to facilitate and augment T cell adhesion to APC [7,8]. In addition, adhesion of T-cells to extracellular matrix proteins is a CD4-regulated process which requires activation by a peptide antigen or mitogen [9–11].

CD4 is also a major receptor protein for the HIV envelope glycoprotein gp120 [12]. Efficient entry of HIV-1 into target cells also requires the chemokine receptors, CCR-5 and CXCR-4, whose interaction with gp120 leads ultimately to viral and cellular membrane fusion [13]. Indeed, binding of CD4 to gp120 greatly increases the efficiency of the gp120-CCR5 interaction, and there is evidence for a physical association between CXCR4 and the CD4-gp120 complex on the cell membrane [14–16]. This association is followed by productive HIV infection in T-cells.

We earlier investigated the interaction of the CD4 receptor with phosphodiester and phosphorothioate oligodeoxynucleotides and we found that oligonucleotides bind to the receptor and interfere with the CD4-gp120 interaction [17]. This in part accounts for the sequence independent anti-HIV activity of oligonucleotides. We have also recently described the ability of CD4, in the presence of [γ-^{32}P]ATP during incubation with human plasma, to undergo phosphorylation at serine and threonine residues [18]. In this paper we report that the extracellular CD4 domain displays a protein kinase activity.

2. Materials and methods

2.1. Materials

Electrophoretic reagents were purchased from Sigma, and molecular weight markers from Sigma and Amersham. Dephosphorylated bovine milk caseins were obtained from Sigma and Merck. [γ-^{32}P]ATP and cold ATP were from Amersham. Chemicals were from Sigma, Aldrich and Fluka.

2.2. Recombinant proteins

The investigation of putative kinase activity of the CD4 receptor was performed using a recombinant extracellular soluble fragment of human CD4 (rsCD4, amino acids 1–362) from Genentech Co. The main experiments on autophosphorylation and on the phosphorylation of casein were reproduced with two rsCD4 samples from Pharmacia-Upjohn and Biogen, with identical results.

2.3. Autophosphorylation of rsCD4

1.5 μg of rsCD4 was incubated with 0.1 mCi of [γ-^{32}P]ATP in 10 μl of 0.05 M Tris-HCl buffer, pH 7.4, at 37°C for 30 min in the absence or presence of 10 mM of divalent cations as chlorides and potential competitors of autophosphorylation (Fig. 1A,B), or 5 μg of rsCD4 were incubated in 10 μl of 150 mM NaCl, 10 mM Tris-HCl, pH 7.4, 1 mM $CaCl_2$ and 0.5 mM $MgCl_2$ with 50 μCi [γ-^{32}P]ATP or [α-^{32}P]ATP, where indicated (Fig. 1C).

2.4. Phosphorylation of substrates

Human plasma was prepared in citrate buffer according to [19]. 10 μl of the prepared plasma was incubated with 0.1 mCi [γ-^{32}P]ATP with and without rsCD4. Bovine milk casein, 10 μg and 5 μg of rsCD4 were incubated with 0.05 mCi [γ-^{32}P]ATP in 10 μl of 10 mM Tris-HCl, pH 7.4, in different salt conditions as indicated in the legend to Fig. 2. After 15 min incubation at 23°C, the reaction was terminated by addition of equal volumes of 20% TCA. The precipitates were washed with acetone and dissolved in 50 mM Tris-HCl buffer, pH 6.8, containing 2% SDS, 2% 2-mercaptoethanol and 20% glycerol. The proteins were analyzed by SDS-electrophoresis.

2.5. Phosphoroamino acid analysis

Two dimensional separation of amino acids of acid hydrolysate of the phosphorylated casein was performed on cellulose TLC plates (Kodak). First direction: electrophoresis (acetic acid/pyridine/water, 87:2:911); second direction: chromatography (*n*-butanol/formic acid/water, 4:1:1) [20].

2.6. Treatment of rsCD4 with diethylpyrocarbonate

5 μg CD4 in 10 μl of 20 mM MOPS buffer, pH 7.0, was incubated for 2 h at 25°C with 0, 1, 5, 10 and 20 mM diethylpyrocarbonate, then Tris-HCl, pH 7.4, was added to a final concentration of 20 mM and incubated for 20 min, then 30 μCi of [γ-^{32}P]ATP in 1 μl was added to the samples with and without 10 μg of casein and they were incubated

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another 20 min at 25°C. Effect on the phosphorylation activity was observed after SDS-PAGE and autoradiography.

2.7. Isolation of autophosphorylated CD4

50 μg rsCD4 and 150 μCi of [γ-^{32}P]ATP were incubated in 30 μl of 20 mM Tris-HCl, pH 7.4, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 70 mM NaCl for 20 min at 37°C and then loaded on a gel filtration column (TSK HW40 Fractogel, Merck, exclusion limit 10 kDa) with a total volume of 250 μl (free vol. about 75 μl). The column was equilibrated with the same buffer as the incubation medium. All 30-μl fractions eluted from the column were collected and the first five fractions were analyzed by incubation in the elution buffer with 15 μg of casein for 30 min at 37°C and separated by SDS-electrophoresis. Maximum radioactivity corresponding to free ATP was eluted in fraction N7.

*2.8. Affinity labeling of rsCD4 with alkylating, radiolabeled ATP derivative (ClRNH-*pppA)*

4-(*N*-2-Chloroethyl-*N*-methyl)aminobenzylamine ($ClRNH_2$) was coupled to γ-phosphate of [γ-^{32}P]ATP in organic solution by reaction with triphenylphosphine/dipyridyl disulfide as previously described [21]. Alkylating grouping $ClRNH_2$ was synthesized in NIBC (Novosibirsk) by Dr. Tamara M. Ivanova. 1 μg of rsCD4 was incubated in 20 μl of 20 mM Tris-HCl, pH 7.4, 0.5 M NaCl, 1 mM CaCl2, 0.5 mM $MgCl_2$ for 40 min at 37°C with increasing concentrations of the alkylating radiolabeled reagent (see Fig. 6).

2.9. SDS-PAGE

Typically 5 μl of 2% SDS, 2% mercaptoethanol, 30% glycerol was added to each 10-μl probe, and the probes were heated in a boiling water bath for 1 min. Then the samples were resolved by SDS-electrophoresis in 12% polyacrylamide; the gels were dried and autoradiographed.

3. Results and discussion

3.1. Autophosphorylation of rsCD4

Fig. 1A shows the results of incubation of rsCD4 with [γ-^{32}P]ATP in the presence of different divalent cations which indicate that the protein undergoes autophosphorylation in the experimental conditions. The efficiency of the process was similar in the absence of added cations, in 10 mM EDTA and in the presence of 10 mM Mg^{2+}, Mn^{2+}, Cu^{2+} and Co^{2+} ions. Ca^{2+} and, to a lesser extent, Zn^{2+} and Ni^{2+}, stimulate autophosphorylation considerably. Fig. 1B demonstrates the effect of some potential substrates and inhibitors (different oligodeoxynucleotides and anionic dyes) on the radiolabeling of rsCD4 in the presence of [γ-^{32}P]ATP and Ca^{2+}. Earlier we found that the CD4 molecule possesses two remote polyanion binding sites, and that the oligodeoxynucleotides longer than 15-mers are capable of spanning the distance between these sites. Both these sites are localized at the amino-terminal domain of the receptor [22,23]. The phosphorothioate oligonucleotide analog SdC28, which displays maximal affinity for the protein (K_d=3 nM) [21], causes maximal inhibition of the protein autophosphorylation (Fig. 1B). Only partial inhibition of autophosphorylation was observed in the presence of the phosphodiester oligodeoxynucleotide pT16 and Active red III dye, the compounds that display a lower affinity for CD4 than the phosphorothioate oligodeoxynucleotide [23,24]. These observations suggest that the phosphorylation activity is localized near the amino-terminal domain of the molecule. We also evaluated mouse IgG as potential inhibitor or substrate of the phosphorylation process (Fig. 1B, lane 1). Total mouse IgG (1 mg/ml) did not affect the autophosphorylation of CD4, and also was not itself phosphorylated.

Pre-heating of the CD4 in solution at 65°C for 10 min

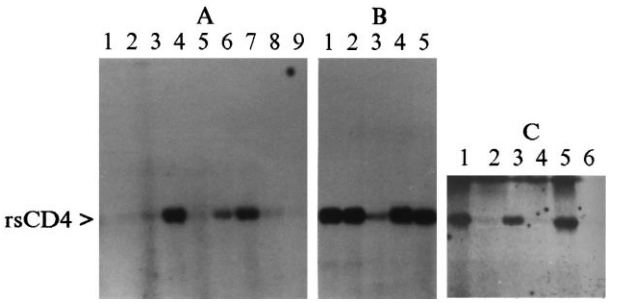

Fig. 1. Effect of some metal ions and potential inhibitors on the autophosphorylation of rsCD4. rsCD4 was incubated with [γ-^{32}P]ATP in the presence of different additives. A: Effect of divalent cations. Lane 1: no additions; lanes 2–9, 10 mM Mg^{2+}, Mn^{2+}, Ca^{2+}, Cu^{2+}, Ni^{2+}, Zn^{2+}, Co^{2+} and EDTA, respectively. B: Effect of potential inhibitors. Lane 1: total mouse IgG (0.3 mg/ml); lane 2: STD, no competitors; lane 3: phosphorothioate oligonucleotide SdC28 (17 μM); lane 4: Active red III dye (17 μM); lane 5: phosphodiester oligonucleotide pT16 (17 μM). C: Autophosphorylation in some specific conditions. Lane 1: 20 min, 25°C; lane 2: the same as lane 1, but CD4 was preheated for 10 min at 65°C; lane 3: 20 min, 4°C; lane 4: the same as lane 1 but with addition of 3 mM of cold ATP; lane 5: the same as lane 1 but 3 mM of unlabeled ATP were added after 20 min of incubation; lane 6: with [α-^{32}P]ATP under the conditions as in sample 1. SDS electrophoresis, autoradiography.

abolishes the formation of radiolabeled products upon incubation with [γ-^{32}P]ATP (Fig. 1C, compare lanes 1 and 2). The process of formation of the ^{32}P-labeled rsCD4 can still occur to some extent even at 4°C (Fig. 1C, lane 3). Addition of excess cold ATP to the reaction mixture inhibits the labeling of the CD4, but addition of the same excess of cold ATP to the mixture after the labeling procedure does not alter the quantity of labeled protein. Finally, substitution of [α-^{32}P]ATP for the [γ-^{32}P]ATP in the reaction mixture abolishes the radiolabeling process (compare lanes 1 and 6 in Fig. 1C), demonstrating that covalently bound γ-phosphate of ATP is responsible for the labeling.

3.2. Phosphorylation of substrates by rsCD4

We then investigated phosphorylation of human plasma proteins in the presence of rsCD4 and [γ-^{32}P]ATP (Fig. 2). No protein radiolabeling can be observed in citrate buffered human plasma incubated with [γ-^{32}P]ATP alone (lane 1). Addition of rsCD4 causes incorporation of the radiolabel into

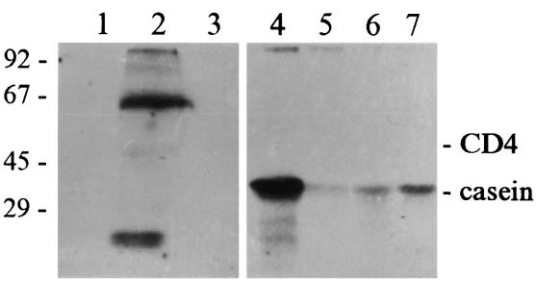

Fig. 2. Phosphorylation of substrates catalyzed by rsCD4. Lane 1: human plasma alone; lane 2: human plasma with rsCD4; lane 3: rsCD4 alone. Lanes 4–7: milk casein with rsCD4 in 10 mM $MgCl_2$ (lane 4); 10 mM $CaCl_2$ (lane 5); 10 mM $MgCl_2$, 150 mM NaCl (lane 6); 10 mM $CaCl_2$, 150 mM NaCl (lane 7). Samples were precipitated with trichloroacetic acid after incubation. For conditions see Section 2. SDS-electrophoresis, autoradiography.

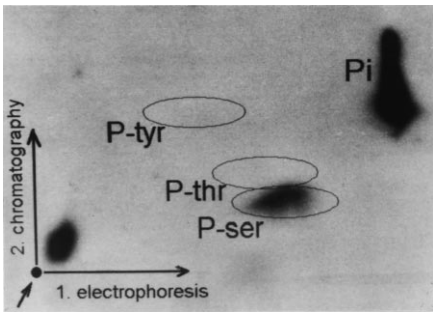

Fig. 3. Amino acid specificity of the phosphorylation of casein with rsCD4 and [γ-^{32}P]ATP (autoradiography).

plasma proteins with molecular masses of 68 kDa and 12 kDa (lane 2). In this experiment, the samples were concentrated with 20% trichloroacetic (TCA) acid before electrophoresis. However, this treatment resulted, surprisingly, in elimination of the label from the autophosphorylated CD4 (lane 3) but not from the labeled plasma proteins (lane 2) suggesting acid lability of the [^{32}P]-CD4 bond. The effect of TCA precipitation on CD4 and substrate casein is also seen in Fig. 4 (compares lanes 1′ at upper and lower panels).

Incubation of rsCD4 and ATP in the presence of dephosphorylated bovine milk casein, a classic substrate for kinase reactions, leads to incorporation of the label into the protein, with formation of an acid-resistant phosphorylation product (Fig. 2). While the presence of Ca^{2+} is required for the formation of the acid-labile complex of [^{32}P]phosphate with CD4, phosphorylation of casein occurs efficiently in the presence of Mg^{2+} at lower ionic strength. At physiologic salt concentration, however, Ca^{2+} supports the kinase reaction better than Mg^{2+}. This dependence on the ionic composition of the buffer of the rsCD4-catalyzed kinase reaction may play a regulatory role in CD4 protein kinase functioning.

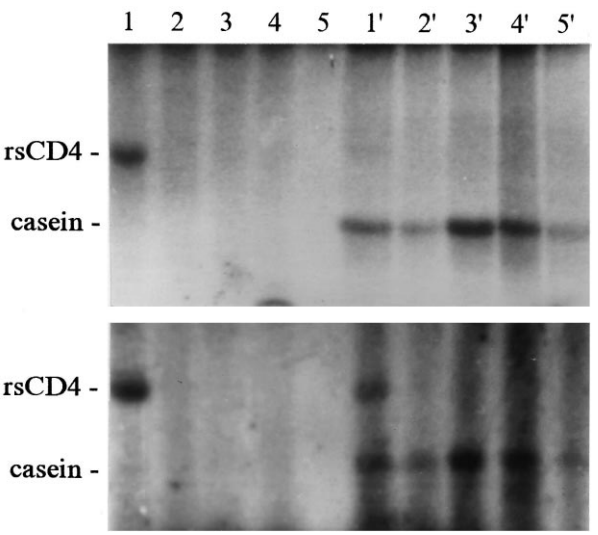

Fig. 4. Effect of diethylpyrocarbonate on phosphorylation activity of rsCD4. Samples 1–5: CD4 was treated with 0, 1, 5, 10, 20 mM diethylpyrocarbonate and then incubated with [γ-^{32}P]ATP (autophosphorylation). Samples 1′–5′: the same as 1–5 except that casein was added to the samples together with [^{32}P]ATP. The upper and lower panels correspond to two almost identical experiments with the difference that samples 1′–5′ on the upper panel were precipitated with trichloroacetic acid, whereas those at the lower panel were not. SDS-electrophoresis and autoradiography.

3.3. Amino acid phosphorylation specificity

Only [^{32}P]phosphoserine (Fig. 3) was detected by phosphoroamino acid analysis [20] after the acidic hydrolysis of the phosphorylated casein. However, analysis of autophosphorylated CD4 detected no phosphoroamino acids in the acidic hydrolysate, which further demonstrates the acid-lability of the [^{32}P]-CD4 bond, thus suggesting that phosphate either forms a phosphoramidate bond with amino groups of lysine, histidine or arginine or it forms acyl-phosphate derivatives with acidic amino acids. These types of bonds are generally assumed for phosphorylated intermediates in enzymatic mechanisms [25]. The autophosphorylation activity yielding acid labile product is associated with participation of His or Tyr residues, because pretreatment of CD4 with diethylpyrocarbonate, a known His and Tyr modifier, inhibits the activity at all concentrations tested. At the same time this reagent even increases the phosphorylation of casein at some concentrations (lanes 3′ and 4′). This means that only His, which forms an acid labile bond with phosphate, may be involved in autophosphorylation as phosphate acceptor, and that phosphorylation of substrate (casein) is likely to be independent of this (autophosphorylation) process. Moreover, the autophosphorylation of CD4 can be inhibited to some extent by the presence of substrate casein (compare lanes 1 and 1′ at the lower panel of Fig. 4). This suggests that the same kinase activity in CD4 molecule may transfer phosphate to its own His or to Ser of substrate and provide both autophosphorylation and phosphorylation processes. Increase in phosphorylation of casein at medium concentration of diethylpyrocarbonate can be explained by the fact that the autophosphorylation reaction is abolished by the His modification and all phosphates bound by the enzyme are used for substrate phosphorylation.

3.4. Possible function of autophosphorylated CD4

We isolated the autophosphorylated labeled CD4 from the reaction mixture using Toyopearl gel filtration media. Then, we found that this ^{32}P-labeled protein which is free of [^{32}P]ATP can phosphorylate substrate casein apparently using the [^{32}P]phosphate incorporated into the protein during autophosphorylation (Fig. 5). It is most likely that the protein has

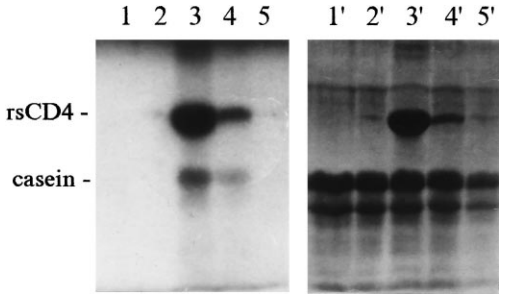

Fig. 5. Phosphorylation of casein by autophosphorylated CD4 in the absence of free ATP. Autophosphorylated CD4 was isolated from reaction medium by gel chromatography (lane numbers correspond to the numbers of chromatographic fractions) and the fractions were incubated with milk casein for 30 min. Left panel (lanes 1–5) is autoradiographed and right panel (lanes 1′–5′) is the corresponding Coomassie stained gel.

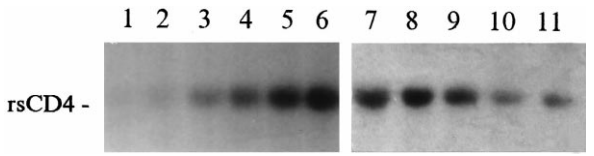

Fig. 6. Affinity labeling of rsCD4 by alkylating ATP derivative. Competition with cold ATP. rsCD4 was incubated with the alkylating radiolabeled reagent ClR-*pppA 0.5, 1, 2.5, 5, 7.5, 10 μM (lanes 1–6, respectively), or with 5 μM of the reagent in the presence of 0, 1, 5, 10, 20 μM of cold ATP (lanes 7–11, respectively). SDS-electrophoresis, autoradiography.

a capacity to accept phosphate for future phosphorylation of biological targets.

3.5. Identification of ATP binding site in rsCD4

We found that the alkylating ^{32}P derivative of ATP, bearing RCl, 4-(*N*-2-chloroethyl-*N*-methyl)aminobenzylamine at the γ-phosphate, forms covalent conjugates with rsCD4 in a concentration dependent manner and that the cold ATP competes with this process (Fig. 6). Affinity of the reactive ATP analog for the CD4 molecule was estimated from the isotherm of chemical modification according to [23] by $K_d \sim 3$ μM. Dissociation constant for cold ATP determined in competition experiment in this affinity labeling reaction according to [22,23] was close to that of the alkylating ATP derivative.

3.6. Homology of CD4 primary structure with protein kinase sequences

Our extensive attempts to find similar regions in the primary sequences of the extracellular domain of the CD4 protein and various protein kinases available from GenBank [26] failed. No homology was detected with near-optimal alignment tools [27], or by a search for protein kinase 'signatures' stored in the PROSITE database [28]. However, we found a weak similarity of the CD4 extracellular domain with myosin light chain kinase sequences (chicken) by means of dot-blot analysis and BLAST searches [29]. Yet we could find no similarities with the known ATP binding sites. Therefore we assumed that the kinase activities of 'conventional' serine-threonine protein kinases and that of the CD4 protein were associated with non-homologous protein structures.

The present study demonstrates that rsCD4 can be autophosphorylated using γ-phosphate of ATP which forms an acid-labile bond with the receptor, probably via His residue. This activity is localized at or near the amino-terminal domain of CD4. The receptor also catalyzes the kinase reaction for casein and blood proteins as a serine/threonine protein kinase. The autophosphorylated CD4 is able to phosphorylate substrate in the absence of ATP in solution operating as a loaded crossbow lying in wait for its target. This protein kinase clearly requires extracellular ATP for its activity. Extracellular ATP is, in fact, necessary for the functioning of T-helper and cytotoxic T-lymphocytes [30]. Moreover an inhibitor of Ser/Thr phosphatase enhances cytolytic T-lymphocyte-mediated cytotoxicity [31]. Thus the CD4 kinase activity may be associated with numerous intermolecular interactions of this molecule.

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