Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography B, 786 (2003) 39–44
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Purification of recombinantly expressed human cluster determinant 4 cytoplasmic domain

Andrea Preusser[a], Gesa Jonas[a], Dieter Willbold[a,b,c,*]

[a]*Institut für Molekulare Biotechnologie, Beutenbergstr. 11, 07745 Jena, Germany*
[b]*Institut für Physikalische Biologie, Heinrich-Heine-Universität, 40225 Düsseldorf, Germany*
[c]*Forschungszentrum Jülich, IBI-2, 52425 Jülich, Germany*

Abstract

A DNA fragment coding for the human CD4 cytoplasmic domain (residues 394–433) was cloned into the pET15b expression vector. The resulting plasmid was used for synthesis of the polyhistidine-tagged $5{\cdot}10^3$ M_r CD4 peptide in *Escherichia coli* BL21(DE3)Star. The CD4 cytoplasmic domain was purified under denaturing and reducing conditions by a two-step procedure using immobilized metal affinity chromatography and gel permeation chromatography. The purified CD4 cytoplasmic domain is soluble and functional without any specific refolding steps. The yield of the described purification procedure was ~5 mg peptide per liter culture volume.

Keywords: Purification; *Escherichia coli*; Cluster determinant 4

1. Introduction

The cluster determinant 4 (CD4) is a type I transmembrane glycoprotein with a molecular mass of $58{\cdot}10^3$ and consists of an extracellular region of 370 amino acids, a short transmembrane region, and a cytoplasmic domain of 40 amino acids at the C-terminal end. The CD4 T-lymphocyte coreceptor (TLC) belongs to the IgG-superfamily and participates in T-cell activation and signal transduction. Surface CD4 is expressed on T lymphocytes that recognize foreign antigens associated with class II major histocompatibility complex (MHC II) molecules [1]. This specificity of $CD4^+$ T cells for MHC II-expressing targets is probably based on direct interaction between CD4 and MHC II [2]. Further, CD4 associates with the T-cell receptor (TCR) during T-cell activation [3]. The mechanism by which CD4 participates in T-cell activation is thought to involve transduction of intracellular signals. Sequence conservation in the cytoplasmic domain of CD4 suggests that this region plays a significant role in this process [4,5]. An explanation for the role of CD4 as a coreceptor for T-cell activation is based on the finding that the lymphocyte specific kinase (Lck) is specifically associated with the cytoplasmic domain of CD4 [6].

In addition to these functions, CD4 serves as the major receptor for human immunodeficiency virus (HIV) infection [7–9]. CD4 interacts via its cytoplasmic domain with viral proteins Nef (negative factor) and Vpu (virus protein U). Vpu induces

*Corresponding author. Present address: Forschungszentrum Jülich, IBI-2, 52425 Jülich, Germany. Tel.: +49-2461-612-100; fax: +49-2461-612-023.
E-mail address: dieter.willbold@uni-duesseldorf.de (D. Willbold).

1570-0232/02/$ – see front matter
doi:10.1016/S1570-0232(02)00731-6

degradation of CD4 molecules resident in the endoplasmatic reticulum. This process requires both proteins to be inserted into the same membrane compartment. The CD4 sequence important in this regard is located between amino acids 402 and 420 [10].

In contrast, Nef acts at the cell surface to mediate the internalization and lysosomal degradation of CD4 [11–13]. Nef dependent downregulation of CD4 is well understood on a cellular level. It appears to involve a whole set of factors [14–21]. From mutational analysis it is known that residues 407–418 in the cytoplasmic domain of CD4 are necessary and sufficient for downregulation of CD4 by Nef. Especially, the dileucine motif at sequence positions 413 and 414 is required for binding and downmodulation of CD4 by Nef [11,12,22–24].

In order to study the interactions of CD4 cytoplasmic domain with cellular and viral interaction partners, e.g. Lck unique domain, HIV-1 Vpu, and HIV Nef, by biophysical methods large amounts of the respective CD4 peptide are needed. Especially, for investigations by nuclear magnetic resonance (NMR) spectroscopy, isotopically labelled CD4 peptide is required. So far, no expression system for CD4 cytoplasmic domain has been described. Thus, we constructed an expression system for production and purification of recombinant CD4 cytoplasmic domain.

2. Experimental

2.1. Materials

Ampicillin (sodium salt, 99% purity) and phenylmethylsulfonyl fluoride (PMSF; 99% purity) were obtained from Carl Roth, Karlsruhe, Germany. 2-Mercaptoethanol (p.a.) was from Merck Eurolab, Darmstadt, Germany. Ubiquitin (bovine red blood cells, 90% purity, 8600 M_r) and bovine insulin (oxidized B chain, 3495.5 M_r) were purchased from Sigma–Aldrich, Taufkirchen, Germany. Bacitracin (1422 M_r), aprotinin (bovine lung, 6511.5 M_r), and ribonuclease A (bovine pancreas, 13 700 M_r) were obtained from Amersham Pharmacia Biotech, Uppsala, Sweden. Biotinylated thrombin, streptavidin-agarose and expression vector pET15b were from Novagen, Madison, USA. ''Complete'' protease inhibitor cocktail (EDTA-free) was from Roche Diagnostics, Mannheim, Germany. Isopropyl-β-D-thiogalactopyranoside (IPTG; 95% purity) was purchased from GERBU Biotechnik, Gaiberg, Germany. Oligonucleotides (HPLC-grade) were obtained from BioTeZ, Berlin, Germany. Restriction enzymes were from MBI, Vilnius, Lithuania. Vent-DNA polymerase was from New England Biolabs, Frankfurt/M., Germany. All other chemicals were of analytical grade.

All buffers and solutions used for peptide purification were degassed by vacuum and supplemented with 7 m*M* (0.05% v/v) or 14 m*M* (0.1% v/v) 2-mercaptoethanol.

2.2. Cloning

2.2.1. Oligonucleotides

Oligonucleotides were as follows: CD4_N5: GTGCGT TCGT TGCCGTC ACCGT CGTCG TCA GGC-TGAACGTATGTCCCAGATCAAAC (56 mer); CD4_C5: GTCTGCTGTCCGAAAAAAAAACCTG-CCAGTGCCCGCACCCGTTTCCAGAAAACCTG-CTCCCCCAGGGG (67 mer); CD4_N3: CAGACG-TTTGATCTGGGACA TACGT TCAGCCTGACGA-CGACGGTGACGGCAACGAACGCACCCC (64 mer); CD4_C3: CTGGGGGAGCAGGTTTTCTGGAAA-CGGTGCGGGCACTGGCAGGTTTTTTTTTCGG-ACAG (59 mer); CD4_Bpu(−): AGGAGGGCTCA-GCTTAGATCGGGGAGCACGTCTTC (35 mer); and CD4_Nde(+): GGAGGACATATGTGCGTTC-GTTGCCGTCACCGTCGTCGTCAGGCTGAACG-TATGTCC (57 mer).

2.2.2. Construction of pET15b_CD4

To generate a synthetic gene coding for CD4 residues 403–433, oligonucleotides CD4_N5, CD4_C5, CD4_N3 and CD4_C3 were annealed and 2 pmol of the annealed oligonucleotide was used as template in a subsequent polymerase chain reaction (PCR) (iCycler, Bio-Rad, München, Germany) employing 50 pmol of oligonucleotides CD4_Nde(+) and CD4_Bpu(−), as well as 3 U Vent-DNA polymerase in 100 μl 1×Vent-polymerase buffer con-

taining 200 μ*M* dNTPs and 2 m*M* $MgSO_4$. The PCR amplification was performed using the following procedure: initial denaturation step, 95 °C for 1 min, 25 cycles with a denaturation step at 95 °C for 30 s, annealing at 50 °C for 30 s, extension at 72 °C for 30 s, following by a final extension at 72 °C for 2 min. The PCR product was separated by electrophoresis in a 2.5% (by mass) agarose gel and extracted (Qiagen gel extraction kit, Qiagen, Hilden, Germany). The resulting CD4 gene flanked by a NdeI and a Bpu1102I recognition site was ligated with NdeI/Bpu1102I restricted pET15b. Ligation product was electroporated into *Escherichia coli* DH5α. The correctness of the CD4 gene was verified by DNA sequencing. Finally, *E. coli* BL21(DE3)Star cells (Invitrogen, Leek, Netherlands) were transformed with plasmid pET15b_CD4 coding for polyhistidine-tagged CD4(394–433) with following amino acid sequence: GSSHHHHHHSSGLVPRGSHMCVRCR-HRRRQAERMSQIKRLLSEKKTCQCPHRFQKTC-SPI.

2.3. DNA sequence analysis

DNA sequence was determined using Big Dye Terminator Cycle Sequencing Kit (PE Applied Biosystems, Warrington, UK) with Ampli Taq DNA polymerase.

2.4. Expression

A 1:50 inoculum of an overnight *E. coli* BL21(DE3)Star *pET15b_CD4* culture in 2 l LB broth (10 g tryptone, 5 g yeast extract, and 5 g NaCl per liter) containing 200 μg/ml ampicillin was allowed to grow aerobically at 37 °C to an $O.D._{600}$ of ~0.8. Expression of CD4(394–433) was then induced with 1 m*M* IPTG. After another 2–3 h of growth at 37 °C, cells were harvested by centrifugation (5000 *g*, 30 min) and sonicated in 20–30 ml extraction buffer (50 m*M* potassium phosphate, pH 7.5, 300 m*M* NaCl, 7 m*M* 2-mercaptoethanol, 3 *M* urea) containing an appropriate amount of protease inhibitor mix (''Complete'', Boehringer Mannheim, one tablet per 50 ml). The extract was clarified by centrifugation (20 000 *g*, 45 min).

2.5. Immobilized metal affinity chromatography

The cell-free extract was applied to a TALON Superflow column (150×10 mm, Clontech, Palo Alto, USA) equilibrated with extraction buffer (50 m*M* potassium phosphate, pH 7.5, 300 m*M* NaCl, 7 m*M* 2-mercaptoethanol, 3 *M* urea). After washing with 6 column volumes of extraction buffer non-specifically bound protein was removed by washing with 3 column volumes of 50 m*M* imidazole in extraction buffer. CD4 peptide was eluted by application of 6 column volumes of 150 m*M* imidazole in extraction buffer. The flow-rate was 2 ml/min.

2.6. Dialysis

CD4 containing fractions, as determined by SDS–PAGE, were pooled. EDTA was added to an end concentration of 5 m*M*. The resulting solution was dialysed twice for 10–12 h in 120-fold volume of water containing 0.5 m*M* HCl and 14 m*M* 2-mercaptoethanol using a Spectra/Por membrane (molecular mass cut-off 1000 M_r; Spectrum Laboratories, Compton, USA). The sample was transferred to a suitable flask, frozen at −80 °C and lyophilized.

2.7. Cleavage

Lyophilized CD4(394–433) was dissolved in 2 ml TBS (50 m*M* Tris–HCl, pH 7.5, 150 m*M* NaCl, 14 m*M* 2-mercaptoethanol). Approximately 3 U of biotinylated thrombin were added and the sample was shaken very carefully at room temperature. After completion of cleavage (2–3 h) thrombin was inhibited by addition of 1 m*M* PMSF and separated from CD4 by addition of streptavidin agarose (50 μl per unit thrombin) and subsequent centrifugation of the streptavidin agarose-biotinyl-thrombin adduct.

2.8. Gel permeation chromatography

Gel permeation chromatography was performed employing a HiLoad Superdex 30 prep grade column (600×26 mm, Amersham Pharmacia Biotech, Uppsala, Sweden) using an ÄKTAexplorer 10S system (Amersham Pharmacia Biotech, Uppsala, Sweden). The peptide was eluted with TBS (50 m*M* Tris–HCl, pH 7.5, 150 m*M* NaCl, 14 m*M* 2-mercaptoethanol)

at a flow-rate of 2.5 ml/min. Absorption was detected at 235 and 280 nm. The column was calibrated with a set of peptides and small proteins (bacitracin $1.4 \cdot 10^3$, insulin $3.5 \cdot 10^3$, aprotinin $6.5 \cdot 10^3$, ubiquitin $8.5 \cdot 10^3$, ribonuclease A $13.7 \cdot 10^3$) with same buffer and flow-rate. Fractions containing CD4(394–433) peptide were dialyzed and lyophilized to be stored at room temperature.

2.9. Tricine-sodium dodecyl sulfate–polyacryamide gel electrophoresis

Tricine-sodium dodecyl sulfate–polyacryamide gel electrophoresis (tricine-SDS–PAGE) was performed on 16.5% slab gels (70×82×7.5 mm) according to Schägger and von Jagow [25]. Gels were stained with Coomassie Brillant Blue [26].

2.10. N-terminal amino acid sequence determination

After tricine-SDS–PAGE the CD4 peptide was blotted onto a Sequi-Blot PVDF membrane (Bio-Rad, Hercules, USA). Western blotting was carried out on a semi-dry blotter (Hoefer SemiPhor, Amersham Pharmacia Biotech, Uppsala, Sweden) using 10 m*M* 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS)/NaOH, pH 11, with 10% (v/v) methanol as transfer buffer [27]. The determination of the N-terminal amino acid sequence was performed with a Protein Sequence System model 494 (Perkin-Elmer Applied Biosystems, Warrington, UK).

3. Results and discussion

The cytoplasmic part of human CD4, despite consisting of only 40 amino acid residues, interacts with a variety of human and viral proteins, such as Lck, IL2-receptor, HIV-1 Nef, and HIV-1 Vpu. Thus, there is tremendous interest in characterization and quantification of the respective interactions on a molecular and atomic level. Preparation of stable isotope labelled CD4(394–433) peptide is a prerequisite for elucidation of the three-dimensional structures of the resulting CD4 complexes by nuclear magnetic resonance (NMR) spectroscopy and for investigation of their dynamic behavior. So far, only chemically synthesized peptides consisting of various portions of CD4 cytoplasmic domain have been available for these kinds of studies.

The hereby described expression and purification procedure for the first time allows preparation of human CD4 cytoplasmic domain from an efficient *E. coli* expression system. The gene coding for the CD4 cytoplasmic domain was designed to ensure optimal codon usage for expression in *E. coli*. Thus, we did not use human cDNA as a template, but rather used synthetic oligonucleotides for construction of the gene coding CD4 cytoplasmic domain. The described purification scheme yielded ~5 mg peptide per liter culture volume.

Reducing and oxygen excluding conditions were necessary to avoid oxidation of cysteine residues being present in this peptide at five sequence positions.

Addition of 3 *M* urea was necessary to ensure effective solubilization of CD4. Without urea a substantial part of the peptide remains in the insoluble fraction of bacterial debris. A further increase in urea concentration did not affect solubility of CD4, but would have inhibited thrombin activity. An additional advantage using 3 *M* urea was elution of CD4 peptide in sharpened peak from metal affinity column (Fig. 1, lane 11). In the absence of urea, elution was extended over a broader fraction range (data not shown). Step-wise elution with imidazole was an efficient method to get an almost pure CD4 peptide. Washing the peptide loaded column with 50 m*M* imidazole led to removal of virtually all non-specifically bound protein (Fig. 1). Urea was removed by dialysis against water that contained 0.5 m*M* HCl and 14 m*M* 2-mercaptoethanol to ensure solubility and to decrease the oxidation tendency of the peptide. No other refolding or renaturing steps were necessary. Identity of the purified fusion peptide was confirmed by MALDI-TOF mass spectroscopy.

Cleavage by thrombin was completed after a few hours (Fig. 2). Thrombin, His-tag and other impurities were removed by gel permeation chromatography (Fig. 3). The CD4 cytoplasmic domain is homogeneous as judged by tricine-SDS–PAGE (Fig. 2, lane 8). Identity of the CD4 peptide was further

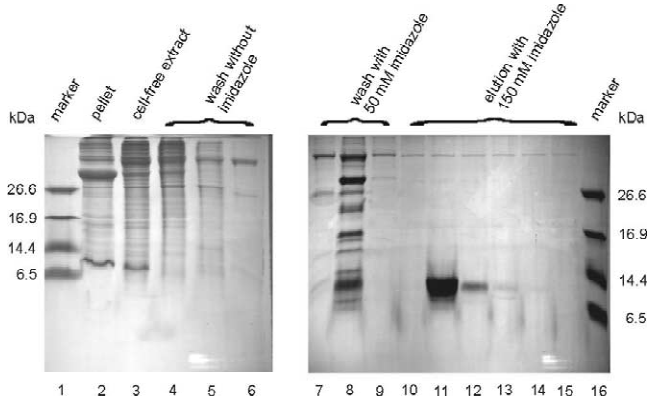

Fig. 1. Result of immobilized metal affinity chromatography of polyhistidine-tagged CD4 cytoplasmic domain on TALON as analyzed by tricine-SDS–PAGE. The fraction size was 12 ml. Aliquots (7.5 μl) of each fraction were applied to 16.5% polyacrylamide gels according to Schägger and von Jagow [25].

confirmed by determination of the amino terminal 17 amino acid residues.

The described expression and purification system of human CD4 cytoplasmic domain allows generation of large amounts of recombinant peptide. The system is also suited to produce isotope labelled CD4 peptide for structural studies by nuclear magnetic resonance (NMR) spectroscopy. The yielded CD4 peptide was fully functional as inferred from a binding assay with uniformly ^{15}N-labelled Lck unique domain employing a ^{15}N-^{1}H-heteronuclear single quantum coherence NMR titration experiment, which will be reported elsewhere.

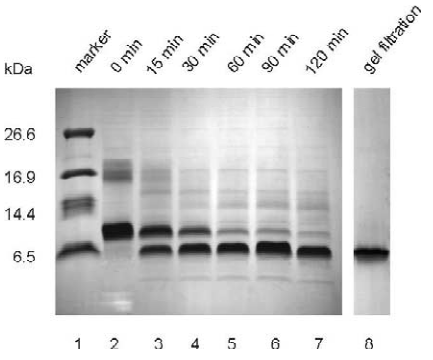

Fig. 2. Analysis of thrombin cleavage of polyhistidine-tagged CD4 cytoplasmic domain by tricine-SDS–PAGE. Progress of cleavage dependent on incubation time is given on top of the lanes. The result after further purification of cleavage products by gel permeation chromatography (Fig. 3) is shown in lane 8.

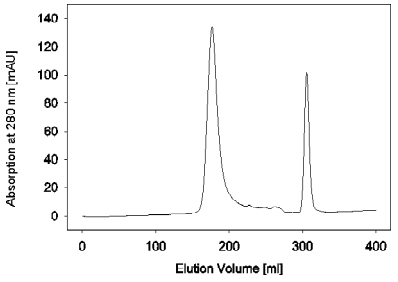

Fig. 3. Result of gel permeation chromatography of thrombin cleavage products of polyhistidine-tagged CD4 cytoplasmic domain on HiLoad Superdex 30. Equilibration and elution buffer was 50 m*M* Tris–HCl, pH 7.5, containing 150 m*M* NaCl and 14 m*M* 2-mercaptoethanol. Shown is the absorbance at 280 nm in dependence of eluted volume. The peak around 177 ml contained the CD4 peptide, whereas the peak at 305 ml did not contain protein.

Acknowledgements

This work was supported by grants from the Deutsche Forschungsgemeinschaft (DFG) to D.W. (SFB466 A4; Wi 1472/4-1). We thank Drs K.-H. Gührs and B. Schlott (Institut für Molekulare

Biotechnologie, Jena) for carrying out mass spectroscopy and N-terminal amino acid sequencing.

References

[1] P.J. Maddon, S.M. Molineaux, D.E. Maddon, K.A. Zimmermann, M. Godfrey, F.W. Alt, L. Chess, R. Axel, Proc. Natl. Acad. Sci. USA 84 (1987) 9155.
[2] W.E. Biddison, P.E. Rao, M.A. Talle, G. Goldstein, S. Shaw, J. Exp. Med. 159 (1984) 783.
[3] W.R. Gallaher, J.M. Ball, R.F. Garry, M.C. Griffin, R.C. Montelaro, AIDS Res. Hum. Retroviruses 5 (1989) 431.
[4] M.C. Miceli, P. von Hoegen, J.R. Parnes, Proc. Natl. Acad. Sci. USA 88 (1991) 2623.
[5] B.P. Sleckman, A. Peterson, J.A. Foran, J.C. Gorga, C.J. Kara, J.L. Strominger, S.J. Burakoff, J.L. Greenstein, J. Immunol. 141 (1988) 49.
[6] A. Veillette, M.A. Bookman, E.M. Horak, J.B. Bolen, Cell 55 (1988) 301.
[7] A.G. Dalgleish, P.C.L. Beverly, P.R. Clapham, D.H. Crawford, M.F. Greaves, R.A. Weiss, Nature 312 (1984) 763.
[8] D. Klatzmann, F. Barre-Sinousi, M.T. Nugeyre, C. Dauguet, E. Vilmer, C. Griscelli, F. Brun-Vezinet, C. Rouzioux, J.C. Gluckman, J.C. Chermann, L. Montagnier, Science 225 (1984) 59.
[9] D. Klatzmann, E. Champagne, S. Chamaret, J. Gruest, D. Guetard, T. Hercend, J.C. Gluckman, L. Montagnier, Nature 312 (1984) 767.
[10] M.Y. Chen, F. Maldarelli, M.K. Karczewski, R.L. Willey, K. Strebel, J. Virol. 67 (1993) 3877.
[11] C. Aiken, J. Konner, N.R. Landau, M.E. Lenburg, D. Trono, Cell 76 (1994) 853.
[12] S.J. Anderson, M. Lenburg, N.R. Landau, J.V. Garcia, J. Virol. 68 (1994) 3092.
[13] A. Sanfridson, B.R. Cullen, C. Doyle, J. Biol. Chem. 269 (1994) 3917.
[14] P.A. Bresnahan, W. Yonemoto, S. Ferrell, D. Williams-Herman, R. Geleziunas, W.C. Greene, Curr. Biol. 8 (1998) 1235.
[15] H.M. Craig, M.W. Pandori, J.C. Guatelli, Proc. Natl. Acad. Sci. USA 95 (1998) 11229.
[16] H.M. Craig, T.R. Reddy, N.L. Riggs, P.P. Dao, J.C. Guatelli, Virology 271 (2000) 9.
[17] M.E. Greenberg, S. Bronson, M. Lock, M. Neumann, G.N. Pavlakis, J. Skowronsky, EMBO J. 16 (1997) 6964.
[18] M. Lock, M.E. Greenberg, A.J. Iafrate, T. Swigut, J. Muench, F. Kirchhoff, N. Shody, J. Skowronsky, EMBO J. 18 (1999) 2722.
[19] S. Benichou, M. Bomsel, M. Bodeus, H. Durand, M. Doute, F. Letourneur, J. Camonis, R. Benarous, J. Biol. Chem. 269 (1994) 30073.
[20] V. Piguet, F. Gu, M. Foti, N. Demaurex, J. Gruenberg, J.L. Carpentier, D. Trono, Cell 97 (1999) 63.
[21] R. Mandic, O.T. Fackler, M. Geyer, T. Linnemann, Y.H. Zheng, B.M. Peterlin, Mol. Biol. Cell 12 (2001) 463.
[22] J.V. Garcia, J. Alfano, A.D. Miller, J. Virol. 67 (1993) 1511.
[23] S. Salghetti, R. Mariani, J. Skowronsky, Proc. Natl. Acad. Sci. USA 92 (1995) 349.
[24] A. Preusser, L. Briese, A.S. Baur, D. Willbold, J. Virol. 75 (2001) 3960.
[25] H. Schägger, G. von Jagow, Anal. Biochem. 166 (1987) 368.
[26] J. Sambrook, E.F. Fritsch, T. Maniatis, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989.
[27] C.S. Baker, M.J. Dunn, Methods Mol. Biol. 32 (1994) 177.