Analysis of Proteins Copurifying with the CD4/lck Complex Using One-Dimensional Polyacrylamide Gel Electrophoresis and Mass Spectrometry: Comparison with Affinity-Tag Based Protein Detection and Evaluation of Different Solubilization Methods

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Mass spectrometry-based identification of the components of affinity purified protein complexes after polyacrylamide gel electrophoresis (PAGE) and in-gel digest has become very popular for the detection of novel protein interactions. As an alternative, the entire protein complex can be subjected to proteolytic cleavage followed by chromatographic separation of the peptides. Based on our earlier report of a method using affinity tag-mediated purification of cysteine-containing peptides to analyse proteins present in an affinity purification of the CD4/lck receptor complex, we here evaluated the use of one-dimensional polyacrylamide gel electrophoresis for analysis of the same receptor complex purification. Using electrospray and tandem mass spectrometry analyses of tryptic peptides from in-gel digested proteins we identified the components of the CD4 receptor complex along with 23 other proteins that were all likely to be non-specifically binding proteins and mainly different from the proteins detected in our previous study. We compare the alternative strategy with the affinity tag-based method that we described earlier and show that the PAGE-based method enables more proteins to be identified. We also evaluated the use of a more stringent lysis buffer for the CD4 purification to minimise non-specific binding and identified 52 proteins along with CD4 in three independent experiments suggesting that the choice of lysis buffer had no significant effect on the extent of non-specific binding. Non-specific binding was inconsistent and involved various types of proteins underlining the importance of reproducibility and control experiments in proteomic studies. (J Am Soc Mass Spectrom 2004, 15, 558–567) © 2004 American Society for Mass Spectrometry

A large number of tools are now available to the proteomics researcher for determining protein identity, relative protein expression or protein interactions in purified protein complexes [1]. In particular mass spectrometry (MS) analysis of the components of purified protein complexes is widely used because identifying new binding partners provides important information on the possible function of any given protein [2]. Using systematic introduction of

affinity-tagged proteins into cells this strategy can be expanded to provide protein-interaction networks aiding in the understanding of biological problems [3]. When purifying protein complexes for MS analysis, several factors need to be considered. The purification strategy needs to aim at preserving the protein interactions that hold the complex together without introducing non-specific binding of abundant cellular proteins. This can be a problem when the use of detergents is required for solubilizing membrane proteins as it has been suggested that different detergents can influence the protein interactions ultimately detected [4]. Furthermore, cross-linking of protein interactions may be helpful to preserve and identify specific protein interactions [5].

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After purification of the complex, the protein mixture needs to be digested with trypsin and the tryptic peptides analysed by MS. Because tryptic cleavage of only a few proteins already produces a large number of peptides, the mixture of which is often difficult to analyse, usually at least one more separation step is required prior to MS analysis. This separation can be achieved via SDS polyacrylamide gel electrophoresis (SDS-PAGE) of the protein mixture followed by analysis of the individual slices [5] or by liquid chromatography-MS (LC-MS) analysis of the tryptic peptides derived from all proteins [6]. Using affinity tags introduced at specific amino acid residues and purification of peptides containing the affinity tag is a way to create a representative subset of only few tryptic peptides per original protein thereby increasing the numbers of proteins that can be detected in one MS experiment [7, 8].

We have reported previously the use of affinity tag-mediated purification of tryptic peptides containing cysteine residues to identify proteins present in an affinity-purification of CD4 [7], a protein involved in T cell activation [9] and HIV entry [10, 11]. We were able to identify the components of the CD4 receptor complex CD4 and lck, a tyrosine kinase that has been reported to associate with CD4 [12] functioning in T cell activation. In addition to those two, we identified several unrelated proteins, which we showed using different control experiments result from non-specific binding. We assumed that non-specific binding was promoted by the use of the non-ionic detergents Triton X-100 and NP-40. Specific protein-protein interactions may have been obscured because of the background from the detergents or ions derived from non-specifically binding proteins. Proteins that did not contain a cysteine residue or where the peptides containing cysteine residues were not detectable with mass spectrometry would also not be identified with that method [7].

In this paper, we report an evaluation of the use of one-dimensional gel electrophoresis followed by mass spectrometry analysis of in-gel digested protein bands as an alternative strategy to identify proteins present in the detergent solubilized sample. We report identification of many different copurifying proteins and provide a comparison between the SDS-PAGE-based method and the previously reported method based on our experience. In order to reduce non-specific binding, we evaluated the use of a detergent combination of NP-40, sodium deoxycholate (DOC) and SDS for cell lysis. This combination was chosen because it has been previously shown that interactions persist in this detergent combination [13] when different subunits of the *N*-methyl-D-aspartate (NMDA) complex could be co-immunoprecipitated. The presence of SDS, which is a potent detergent for disrupting non-covalent protein interactions suggests that weak protein interactions such as non-specific binding will be reduced. We therefore chose to compare the extent of non-specific binding when using this lysis buffer with the non-specific bind-

ing we detected when Triton/NP-40 was used for cell lysis.

Experimental

Unless otherwise specified, all chemicals and reagents were obtained from Sigma (St Louis, MO). PolyT4-5 antibody was a generous gift from Dr Ray Sweet (Smith-Kline Beecham, King of Prussia, PA). Q425 antibody was purified from hybridoma supernatant and conjugated to cyanogen bromide activated sepharose according to the manufacturer's recommendation. Q425 antibody secreting hybridoma cells had been obtained from Dr Quentin Sattentau (Center D'Immunologie de Marseille-Luminy, Marseille, France).

Tissue Culture and Affinity Purification

The lymphoblastoid cell line CemT4 was obtained from the NIH AIDS Research and Reference Reagent Program (Rockville, MD). Cells were cultivated in RF-10 medium (CSL, Parkville, Victoria, Australia) to a total of $\sim 10^9$ cells. Cell lysate preparation and affinity purification was performed similarly as described previously [14]. Briefly, cells were grown to a total amount of 1×10^9 cells and lysed in either lysis Buffer 1 or 2. For lysis in Buffer 1, cells were washed twice with ice cold phosphate-buffered saline (PBS) and lysed in HEPES-buffered saline (HBS), 1% (vol/vol) Triton X-100, 1% (vol/vol) NP-40, 10 mM MgCl_2 and a cocktail of the protease inhibitors phenylmethylsulfonylfluoride, aprotinin and leupeptin at a concentration of 10^7 cells/mL. For lysis in Buffer 2, cells washed twice with ice cold HBS, pH 8.0, 1 mM MgCl_2 , 0.1 mM [ethylenedis(oxyethylenetrinitrio)]tetraacetic acid (EGTA) and 0.02% (wt/vol) NaN_3 . Cells were then lysed in HBS, pH 8.0, 1 mM MgCl_2 , 0.1 mM EGTA, 0.02% (wt/vol) NaN_3 , 1% (vol/vol) NP-40, 0.5% (wt/vol) DOC and 0.1% (wt/vol) SDS at a concentration of 10^7 cells/mL. After a 1 h incubation at 4 °C insoluble material was pelleted at $10,000 \times g$ and the supernatant was passed through a $0.22 \mu\text{m}$ sterile filter (Millipore, Bedford, MA) and supplemented with 10 mM CaCl_2 . The lysate was then applied to a Q425 affinity column that had been equilibrated with HBS, 1% (vol/vol) Triton X-100, 1% (vol/vol) NP-40 and 10 mM CaCl_2 . A separate column was used for each lysis buffer. The column was washed with 50 mL tris-buffered saline (TBS), pH 8.0, 1% (vol/vol) Triton X-100, 1% (vol/vol) NP-40 and 10 mM CaCl_2 followed by 20 mL of TBS, pH 8.0, 1% (vol/vol) Triton X-100, 1% (vol/vol) NP-40. Proteins were then eluted with TBS, pH 8.0 containing 10 mM ethylenediaminetetraacetic acid (EDTA) and 0.1% (vol/vol) Triton X-100 and 0.1% (vol/vol) NP-40. Elution fractions were tested for CD4 using poly T4-5 antibody and Donkey anti rabbit coupled to horseradish peroxidase (HRP) (Amersham-Pharmacia, Uppsala, Sweden).

Figure 1. Visualization of proteins present in the affinity-purification of the CD4 receptor complex. CD4 and associating proteins from approximately 10^9 cells were separated on an SDS-Polyacrylamide gel and stained with Coomassie Brilliant Blue.

SDS-PAGE and Coomassie Brilliant Blue Staining

For SDS-PAGE the two to three elution fractions containing the majority of CD4 were combined and 100 μ L of Strataclean resin (Stratagene, La Jolla, CA) was added. Proteins were allowed to bind to the resin for 2 h at 4 °C. The resin was then pelleted and proteins were eluted with 2 $\times$ 30 μ L sample buffer (62.5 mM Tris, pH 6.8, 0.025% (wt/vol) bromophenol blue, 10% (vol/vol) glycerol, 2% (wt/vol) SDS and 25 mM tris[2-carboxyethyl]phosphine hydrochloride (TCEP) (Pierce, Rockford, IL) at 95 °C. The eluate (50 μ L) was loaded onto a 8–16% gradient gel (Gradipore, Frenchs Forest, NSW, Australia) and proteins were separated with a constant current of 25 mA per gel. Proteins were stained with Coomassie Brilliant Blue as described [14] and destained in 40% (vol/vol) methanol, 10% (vol/vol) acetic acid.

In-Gel Digest and Peptide Extraction

Excised gel-slices were dehydrated with methanol for 5 min and rehydrated with 30% methanol for 5 min. Slices were washed twice with water and then three times 10 min with 100 mM ammoniumbicarbonate, 30% (vol/vol) acetonitrile. Gel slices were then crushed into pieces and dehydrated for 5 min with 100% ethanol. Ethanol was removed and the pieces were dried for 1 h in vacuum. Depending on the original size of the gel-slice, 50–80 μ L of trypsin solution (5 ng/ μ L sequencing grade modified trypsin (Promega, Madison, WI) in 25 mM ammoniumbicarbonate solution) were added and the protein hydrolysis was carried out at 37 °C over night. The supernatant was aspirated and proteins were extracted three times by addition of 50 μ L

extraction solution (50% acetonitrile, 5% formic acid) for 30 min. In between extractions, gel-slices were rehydrated by addition of 30 μ L water and 20 min incubation. The aspirated extraction solutions and water supernatants were combined with the aspirated trypsin solution and solvents were evaporated using a Speed-Vac centrifuge with moderate heating until $\sim$ 20 μ L remained. Water (20 μ L) was added twice to ensure that the remaining solution was free of acetonitrile. Peptides were purified for MS analysis using ZipTips (Millipore, Bedford, MD) according to the manufacturer's recommendations. Samples were acidified before purification by addition of 15 μ L 5% formic acid and trifluoroacetic acid was replaced by 5% formic acid throughout the purification.

Mass Spectrometry Analysis

Electrospray mass spectrometry (ESI-MS) analyses were carried out as previously described [7] using a Quadrupole-Time-of-flight (Q-TOF) 2 instrument (Micromass, Manchester, UK) in nanoelectrospray mode. Typically 5–8 μ L sample were loaded into a coated glass capillary (Protana, Odense, Denmark). The ESI parameters were as follows: Capillary voltage, 900–1200 V; Cone voltage, 30–40 V; desolvation temperature, 40 °C; desolvation gas flow rate, 50–100 l/h. Peptides were detected by acquisition of the m/z range from 50–2000 using a 2.4 s integration time and a 0.1 s delay. Precursor ions were selected for fragmentation within a mass window of 4 m/z . All multiply charged ions that were clearly distinguishable from the background were selected for fragmentation. MS/MS spectra were collected using collision cell voltages from 25 V to 35 V with argon as the collision gas. The instrument was calibrated with a solution of 100 fM [Glu¹]-Fibrinopeptide B in 40% acetonitrile, 2.5% formic acid. To reduce the intensity of detergent-derived singly charged species, the multichannel plate (MCP) detector voltage on the detector was reduced to 2000 V during MS acquisitions whilst kept at 2300 V during MS/MS acquisitions [15].

Protein Identification

Spectra were interpreted and matched using either the MASCOT search engine (http://www.matrixscience.com/cgi/index.pl?page=/search_form_select.html) or by manual de-novo sequencing. Manual sequencing was also used to confirm all peptide matches obtained via MASCOT and performed similarly as described before [7]. Amino acid sequence tags obtained from the MS/MS spectra were used for database searches with the PepSea software (Protana, Odense, Denmark, <http://195.41.108.38/PepSeaIntro.html>) or the ScanProsite software (SWISS-PROT database, Swiss Institute of Bioinformatics (SIB), Geneva, Switzerland, <http://au.expasy.org/tools/scanprosite/>). Proteins containing the sequence tag were subjected to a theoretical digest with trypsin and the resulting peptides searched for the

Table 1. Proteins identified by ESI-MS/MS in all experiments

Protein name	Spot # ^a	MW kDa	Number of Cys	Peptides identified in experiment					
				[7] ^b	1A ^c	1B	2A	2B	2C
Leucine-rich protein	P42704	145	21	.	.	.	8	.	.
Eukaryotic translation initiation factor 3 subunit 8	Q99613	105	12	.	.	2	.	.	..
Importin β -2 subunit	Q92973	101	26	.	.	.	1	.	.
Importin β -1 subunit	Q14974	97	23	.	.	.	3	.	.
Eukaryotic translation initiation factor 3 subunit 9	P55884	92	7	.	.	1	.	.	.
Heat-shock protein HSP 90- α	P07900	85	7	.	.	.	3	.	.
Neprilysin	P08473	85	12	.	.	.	.	1	.
Heat-shock protein HSP 90- β	P08238	83	6	.	.	.	8	.	.
Structure-specific recognition protein 1	Q08945	81	6	.	2	4	.	.	4
Annexin A6	P08133	76	6	.	.	.	.	9	11
Nucleolin	P19338	76	1	.	.	5	.	.	5
DEAD-box protein 3	O00571	73	7	.	.	1	.	.	.
Heat shock cognate 71 kDa protein	P11142	71	4	.	4	2	3	.	.
Probable RNA-dependent helicase p68	P17844	69	9	.	.	2	.	.	.
Phenylalanyl-tRNA synthetase β chain	Q9NSD9	66	11	.	.	1	.	.	.
Eukaryotic translation initiation factor 3 subunit 7	O15371	64	11	.	.	4	.	.	.
60 kDa Heat shock protein	P10809	58	3	.	.	.	4	.	.
P56lck	P06239	58	9	1	2	2	9	16	2 ^d
ATP synthase α chain, mitochondrial	P25705	55	2	.	.	.	3	.	.
CD4	P01730	55	11	2	3	1	3	7	5 ^d
Vimentin	P08670	54	1	.	.	.	1	.	.
Eukaryotic translation initiation factor 3 subunit 6	Q64252	52	7	.	2	6	.	.	.
Tubulin α -1 chain	P05209	50	12	.	.	1	.	.	.
Tubulin β -1 chain	P07437	50	9	.	2 ^e	1	.	.	.
Heterogeneous nuclear ribonucleoprotein H	P31943	49	5	.	.	1	.	.	.
α enolase	P06733	47	6	.	.	.	8	.	.
Eukaryotic initiation factor 4A-II	Q14240	46	4	.	.	.	1	.	.
GA17 protein	O60735	43	8	.	1	.	.	.	.
Actin, cytoplasmic I	P02570	42	6	.	.	.	2	.	.
Guanine nucleotide-binding protein G(i), α -2 subunit	P04899	40	10	.	.	.	.	.	1
Annexin A1	P04083	39	4	.	.	.	.	2	2
Heterogeneous nuclear ribonucleoprotein A1	P09651	39	2	.	.	1	1	.	.
Annexin A2	P07355	38	4	.	.	.	.	8	7
Heterogeneous nuclear Ribonucleoprotein D0	Q14103	38	3	.	.	1	.	.	.
Voltage-dependent anion-selective channel protein 2	P45880	38	10	.	.	.	1	.	.
Heterogeneous nuclear ribonucleoproteins A2/B1	P22626	37	1	.	.	1	.	.	.
Eukaryotic translation initiation factor 3 subunit 2	Q13347	37	7	1	1	.	.	.	.
Glyceraldehyde 3-phosphate dehydrogenase	P04406	36	3	.	.	.	6	2	.
Nuclease sensitive element binding protein 1	P16991	36	0	.	.	.	.	.	2
Guanine nucleotide binding protein	P25388	35	8	3	.	.	.	.	.
60 S acidic ribosomal protein P0	P05388	34	3	.	.	2	.	.	1
40 S ribosomal protein SA	P08865	33	2	.	.	4	.	.	.
Nucleophosmin	P06748	33	3	.	.	1	.	.	.
Voltage-dependent anion-selective channel protein 1	P21796	31	2	.	.	.	1	.	.
40 S ribosomal protein S3A	P49241	30	4	.	.	1	1	.	.
60 S ribosomal protein L7A	P11518	30	3	.	.	.	.	.	1
Voltage-dependent anion-selective channel protein 3	Q9Y277	30	6	.	.	.	1	.	.
Prohibitin	P35232	30	1	.	.	.	2	.	.
40 S ribosomal protein S4	P12750	29	4	.	.	.	.	.	1
60 S ribosomal protein L7	P18124	29	1	.	.	.	.	.	1
Dehydrogenase/reductase SDR family member 2	Q13268	27	6	.	.	.	2	.	.
Elongation factor 1- β	P24534	25	3	.	.	.	2	.	.
HSPC029	Q9Y6D1	25	5	2	.	.	.	.	.
40 S ribosomal protein S8	P09058	24	5	.	.	.	.	.	1
GTP-binding nuclear protein RAN	P17080	24	3	.	.	.	1	1	.
40 S ribosomal protein S7	P23821	22	0	.	.	.	.	.	1
40 S ribosomal protein S9	P46781	22	1	.	.	.	.	.	1
60 S ribosomal protein L18	Q07020	22	2	.	.	.	.	.	1
60 S ribosomal protein L11	P39026	20	4	.	.	.	.	.	1
Myosin regulatory light chain	P19105	20	1	1	.	.	.	.	.
40 S ribosomal protein S10	P46783	19	0	.	.	.	.	.	1
40 S ribosomal protein S18	P25232	18	0	.	.	.	.	.	4

(Continued)

Table 1. (Continued)

Protein name	Sprot # ^a	MW kDa	Number of Cys	Peptides identified in experiment					
				[7] ^b	1A ^c	1B	2A	2B	2C
40 S ribosomal protein S13	Q02546	17	0	.	.	.	.	.	1
Myosin essential light chain	P16475	17	3	1	.	.	.	.	.
40 S ribosomal protein S19	P39019	16	0	.	.	.	.	.	1
40 S ribosomal protein S24	P16632	15	0	.	.	.	.	.	1
60 S ribosomal protein L22	P35268	15	1	.	.	.	.	.	1
40 S ribosomal protein S12	P25398	14	6	1	.	.	.	.	.
Histone 2B	P02278 ^f	14	0	.	.	.	.	.	2
60 S Acidic Ribosomal Protein P1	P05386	12	2	2	.	.	.	.	.
Ubiquitin	P02248	8.5	0	.	.	.	.	.	1
Total: 66 proteins					8	23	26	8	26

^aSwiss-Prot accession number^bDetected in our previous study[7]^c+ Indicates in which of five experiments the protein was detected. Experiment labels 1/2 refer to the respective lysis buffer Triton X-100/NP-40 or NP-40/DOC/SDS and A/B/C refer to one of two or three independent experiments.^dSeveral ions typical of CD4- or lck-derived peptides were detected but not subjected to fragmentation.^ethe detected peptide matched two or more tubulin beta chains^fthe detected peptide matched various alleles of the same protein, one allele is listed representative for all matches.

peptide that contained the sequence tag. To be identified as the sequence of a fragmented ion, the peptide mass needed to be equivalent to the experimentally obtained mass including modifications such as oxidized methionine residues or carboxyamidomethylcysteine residues. Additionally, a series of product ions from the MS/MS spectrum had to match the predicted y and b ions of the sequence requiring a mass accuracy of 0.1% for both the masses of product ions and the mass differences between product ions representing amino acids. The full peptide sequence was again entered into the ScanProsite search engine and the protein was considered to be identified conclusively in the affinity chromatography-derived sample if it was the only human protein producing this peptide after a tryptic digest.

Results

Proteins Detected Visually in the Coomassie Stained Gels

To identify proteins purifying together with CD4, the affinity-purified complexes from $\sim 1 \times 10^9$ cells were concentrated and separated on an 8–16% gradient polyacrylamide gel. Figure 1 shows a representative example for all experiments of a Coomassie stained gel containing the proteins of a CD4 affinity purification using the SDS containing lysis buffer. The visible pattern is very weak and apart from major bands at 60, 50, and 37 kDa, several minor bands of different strengths (but all very weak) can be seen.

Proteins Identified in the Sample Prepared with Triton X-100/NP-40 Lysis Buffer

Two experiments were carried out to determine proteins present in an affinity purification of CD4 employing the Triton X-100/NP-40 based lysis buffer. Whereas

in the first experiment, only visible protein bands were excised and analysed, in the second series of experiments, the entire lane of the gel was dissected into slices from the very top of the gel down to the area representing the molecular weight of 15 kDa. A total of 23 proteins were identified in the two different experiments using the 1D-PAGE-MS strategy. Table 1 lists all identified proteins. In all, 8 proteins were identified in the first experiment, 6 of them were identified again in the second experiment along with 15 other proteins.

Proteins Identified in the Sample Prepared with NP-40/DOC/SDS Lysis Buffer

To investigate whether the use of a more stringent detergent combination has a positive effect on reducing non-specific binding, three experiments were carried out employing a different detergent combination including SDS in the lysis buffer. CD4 complexes were purified as described above and the presence of CD4 in lysate and elution fractions but not in the flow-through fraction of the affinity column was confirmed (data not shown) to ensure that CD4 binding to the antibody is not abrogated in the presence of SDS. Figure 2 shows a representative mass spectrometry analysis of the purified peptide mixture obtained from a tryptic digest of an excised band containing only a barely visible amount of protein. The inset shows the expansion of a part of the spectrum allowing the identification of doubly charged ions at m/z 802.6 and 803.6. Figure 3 shows a MS/MS spectrum of the ion at m/z 802.6 leading to the unambiguous identification of elongation factor 1- β . Sixteen peptide ions were identified in this gel slice as indicated with the labels resulting in the identification of 9 different proteins (excluding trypsin). A total of 54 proteins were identified in three independent experiments using 1D-PAGE MS analysis of purified CD4 derived from cells lysed with NP-40/DOC/SDS buffer.

Table 1 lists all proteins that were identified in these experiments.

Discussion

Proteins usually carry out their function in complexes and the knowledge of the interacting partners is crucial to determine the function of a given protein. CD4 has multiple functions in T cell activation [9], as an interleukin-16 receptor [16] and in HIV-1 entry [10, 11]. We have investigated previously proteins copurifying with CD4 in search for novel binding partners that could play a role in the function of this receptor [7]. In that study proteins were identified using affinity tag-mediated purification of tryptic peptides carrying a biotin tag at cysteine residues. However, we were only able to identify the CD4/lck complex along with several non-specifically binding proteins. We suggested that proteins present in the affinity chromatography-derived sample could have escaped detection because they did not contain a cysteine residue required for biotin labeling or the labeled peptide was not detectable via ESI-MS analysis [7]. To investigate the possibility that potential protein partners were missed, in this study we evaluated the use of SDS-PAGE followed by in-gel digest and mass spectrometry analysis as an alternative method to the affinity tag-mediated purification of biotin labeled peptides reported in our previous study [7]. SDS-PAGE has the advantage that the protein pattern can be visualised with staining and that individual bands can be excised and the approximate molecular weight assists in their later identification. As Figure 1 shows, in all our experiments, only a very faint pattern of proteins was visible after the gel was stained showing that affinity purification of CD4 from lymphoid cells produces only a small amount of protein, roughly estimated less than 1 μ g from the intensity of the band. Still, the employed strategy led to the identification of many CD4-derived peptides for which clean and easy-to-interpret MS/MS spectra were obtained. The same applies to the tyrosine kinase lck. Most of the CD4 is associated with lck in lymphoid cells [12], so the purification strategy led to the isolation of a similar amount of lck so that this protein was identified as easily as CD4.

Besides a band at ~55 kDa, that could incorporate either or both CD4 (55 kDa) and lck (58 kDa), several other albeit often barely visible bands were present in the protein pattern. Analysis of gel slices containing those proteins was still able to unambiguously identify the proteins that were contained in the slices. Even regions of the gel that did not contain any visible protein band yielded protein data upon mass spectrometry analysis demonstrating the ability of the strategy to detect proteins in sub Coomassie stainable amounts (i.e., <pmols). In some instances, up to nine different proteins along with trypsin could be identified in one mass spectrometry analysis of a single gel slice that

contained only a barely detectable amount of protein (Figures 2 and 3, Table 2).

A total of 23 different proteins were identified in two different experiments in the affinity purification of CD4 employing similar purification methods as in our previous report [7]. However, in that study, we were only able to detect a total of 10 proteins in three different experiments indicating that the SDS-PAGE based method is a more powerful approach towards identifying most of the proteins present. CD4 and lck were detected in all performed experiments, but only two other proteins were identified by both technological approaches. Significant run-to-run differences in the intensity of non-specific binding to CD4 probably account for much of these differences but the differential abilities of the two methods to identify proteins is also a factor. Whereas the SDS-PAGE based method can identify a protein based on any of its tryptic peptides, the affinity tag-based method specifically detects only peptides containing a cysteine. As not all proteins contain a cysteine residue and not all peptides containing a cysteine residue can be observed in a mass spectrometry experiment [7], the SDS-PAGE based method has the potential to identify more of the proteins present in the sample. Of the 66 proteins we identified in this study, 9 do not contain a cysteine residue and therefore would have been missed using the affinity tag-mediated purification. In contrast, non-PAGE based methods have the advantage of identifying proteins which migrate poorly in gels [17] and the detection of the small acidic myosin and ribosomal proteins in the affinity tag-based method could be because this method does not involve such a separation. Therefore the observed difference in the identified proteins is likely to be a combination of both the differential protein binding in different experiments, the different sensitivities of the methods but also the lower overall sensitivity of the affinity tag-based method because of the interference by the detergents.

When choosing the appropriate method for an investigation, several other factors must be taken into account concerning the speed with which the experiments and the protein identification can be carried out. Protein identification fundamentally relies on how efficiently tandem mass spectra can be matched to tryptic peptides of proteins. Whereas the affinity tag-based method led to detection of mainly peptides of high molecular weight [7], which were often difficult to match, the gel-based method mainly detected peptides in the mass range of 1000 to 2000 Da which is more convenient for manual analysis. Also the samples were less contaminated with singly charged species as the SDS-PAGE separation eliminated the non-ionic detergents that caused a significant background in the affinity tag-based method [7]. The time for sample preparation is another factor. The affinity tag-based method has the advantage that representative peptides obtained from all proteins are

Figure 2. Mass spectrometry identification of peptides derived from the tryptic digest of an excised gel slice. The ESI-mass spectrum of the tryptic peptides derived from the proteins in a gel slice of about 30 kDa is shown together with the ions at m/z 802.6 and 803.6 enlarged.

present in a single fraction, which is relatively easy to investigate. Moreover the SDS-PAGE based method leads to a high degree of redundant protein identification. More than 15 peptides have been observed for lck for example (Table 1). Also each gel slice needs to be analysed separately leading to a much more time-consuming analysis when using this method. Nevertheless the investigation of slices, provides the analyst with the approximate molecular weight of the protein (as noted above), which can be an advantage when doing a database search for protein identification. Clearly this study demonstrates that no one method is capable of identifying all proteins present in such a complex mixture. Each method has its particular advantages, however for our aims the

SDS-PAGE based method seemed more efficient because more proteins were detected and the spectra obtained were generally cleaner. When choosing the appropriate method for an investigation, those factors need to be taken into account and to achieve detection of as many proteins as possible, the use of both strategies may be necessary.

Because of the high degree of non-specific binding to CD4 detected in our earlier study, we aimed to evaluate a different purification strategy that reduces the non-specific binding to CD4. Also, a part of CD4 has been reported to participate in detergent-insoluble microdomains [18] and this compartment together with any possible associating molecules, is likely to escape detection in a method employing cell lysis in

Figure 3. Identification of proteins via tandem mass spectrometry. The MS/MS spectrum of the ion at m/z 802.6 ($2+$, precursor mass 1604.2 Da) is shown. Singly charged product ions are labeled. The peptide sequence SPAGLQVLNDYLADK identified it as derived from the elongation factor 1- β .

Table 2. Peptides and proteins identified in an excised gel-slice of ~30 kDa

Precursor <i>m/z</i> ^a	Peptide mass ^b	Sequence ^c	MASCOT Score	Protein Name	Swiss Prot#	MW kDa
473.4	944.6	LVPVGYGIK	12	Elongation factor 1-β	P24534	24.6
501.4	1001.5	LITEDVQ GK	25	40 S ribosomal protein S3A	P49241	29.8
508.9	1015.6	LTLSALVDGK	34	Voltage-dependent anion-selective channel protein 2	P45880	38.1
575.4	1029.6	LTLSALIDGK	33	Voltage-dependent anion-selective channel protein 3	Q9Y277	30.7
593.4	1184.7	DLQNVNITLR	26	Prohibition	P35232	29.8
607.5	1212.7	VLPSITTEILK	34	Prohibition	P35232	29.8
618.0	1233.7	IEVIEIM*TDR	29	Heterogeneous nuclear ribonucleoprotein A1	P09651	38.7
687.5	1373.7	WTEYGLTTFEK	7	Voltage-dependent anion-selective channel protein 1	P21796	30.6
706.5	1410.8	GALQNIIPASTGAAK	32	Glyceraldehyde 3-phosphate dehydrogenase	P04406	35.9
737.8	2210.1	LGEHNIDVLEGNEQFINAAK	47	Trypsin	P00761	23.5
749.2	2244.1	VIISAPSADAPM*FVM*GVNHEK	13	Glyceraldehyde 3-phosphate dehydrogenase	P04406	35.9
802.6	1602.8	SPAGLQVLNDYLADK	57	Elongation factor 1-β	P24534	24.6
803.6	1604.9	VAVVTGSTSGIGFAIAR	17	Dehydrogenase/reductase SDR family member 2	Q13268	27.3
807.6	1612.9	LVINGNPITIFQER	46	Glyceraldehyde 3-phosphate dehydrogenase	P04406	35.9
882.6	1762.8	LISWYDNEFGYSNR	42	Glyceraldehyde 3-phosphate dehydrogenase	P04406	35.9
931.1	1860.0	SPALLSQQLLPYM*ENR	45	Dehydrogenase/reductase SDR family member 2	Q13268	27.3

^aGiven is the experimentally determined *m/z* value.^bGiven is the theoretical uncharged monoisotopic peptide mass.^cM* indicates oxidized methionine.

cold Triton X-100. To reduce contamination with abundant proteins from the transcription/translation machinery and to generally increase solubility of membrane proteins a detergent combination was used for cell lysis that had been reported to efficiently solubilize proteins from the NMDA receptor complex [19]. Co-immunoprecipitation studies on the NMDA receptor complex have demonstrated that interactions between different subunits of the complex persist even in the presence of up to 0.2% SDS [13, 20]. However, SDS is a potent detergent for inhibiting noncovalent protein-protein interactions and its incorporation should reduce non-specific protein interactions. Table 1 lists all proteins identified in three independent experiments, in which a total of 49 proteins were identified together with CD4 and Ick. Only these two were identified in all experiments. Otherwise the pattern of copurifying proteins is different between the individual experiments and also different from the experiments employing the Triton X-100/NP-40 lysis buffer. However, there was no obvious reduction in non-specific binding and any conclusions on the nature of binding proteins in relation to the employed lysis buffer cannot be drawn because of the variability between the experiments. Based on these data, it cannot be stated whether employing the NP-40/DOC/SDS lysis buffer has any beneficial influence on non-specific protein binding. It could very well be that it does decrease non-specific binding but also induces partial unfolding of CD4 and/or Ick-domains that are then accessible to non-specific binding, counteracting such benefits derived. Thus the choice of lysis buffer in this experiment has no significant influence on purification of both the CD4 receptor complex and on non-specific binding.

Whereas our strategy aimed to reduce non-specific binding by using SDS as a detergent for cell lysis, tandem affinity purification (TAP) would be an alternative way of reducing the presence of non-specifically binding proteins when purifying complexes for mass spectrometry analysis. It is normally performed by recombinantly expressing the protein of interest containing two different affinity tags with which two different affinity purifications are performed. The sequential purification leads to a high purity of the complex increasing the chance that detected proteins are specific interactors. It has been successfully used for several proteomic studies including for example the human Smad proteins [21]. Those strategies are especially efficient when complexes are purified that contain large numbers of subunits that provide a big platform for non-specific binding. In our study it could have been performed using two different monoclonal antibodies for affinity-purification of CD4, even using two columns with the same antibody would have been an option. Alternatively, CD4 could have been expressed as a recombinant tagged protein such as (His)₆-tagged in a CD4-negative cell line like A2.01 and TAP carried out via Ni-NTA and Q425 affinity chromatography. However, some preliminary studies have shown difficulties when eluting CD4 off columns made of other immobilised CD4 antibodies such as OKT4 (data not shown). Recombinantly expressing tagged CD4 is also rather laborious so we have considered tandem-affinity purification to be a method of choice if the results obtained in this study had shown evidence that a large complex were present. As no evidence of a large CD4-containing complex could be found, we did not evaluate this option further.

Eight proteins were found to copurify with CD4 in

our previous report [7] but none of them was functionally related to CD4 and using control experiments, we could show that all of them are non-specifically binding proteins. In this study, we detected a further 62 proteins. As none of the detected proteins showed consistent signals in all experiments as observed for p56lck, the detected proteins were unlikely to be functionally related to CD4, similarly to the proteins detected in our previous study [7]. Therefore no experiments were conducted to test the specificity of the interaction. This could have been done using independent technologies or by conducting control experiments with non-specifically absorbing beads as we [7] and others [2] have shown. Along with the vast majority of the detected proteins, which are derived from the abundant protein synthesis, transcription or RNA processing machinery, several proteins from the cytoskeleton (actin, tubulin, and vimentin) were detected. As we have previously stated, a connection between CD4 and the cytoskeleton has been reported and the detection of components of the cytoskeleton could be a result thereof [22, 23]. However, more investigation is required to further clarify the mechanism of an association between CD4 and the cytoskeleton.

In conclusion, this study shows that neither of the above-investigated strategies is superior in detecting the proteins present in the affinity-chromatography-derived sample. Given the large number of non-specifically binding proteins and the different protein families they belong to it also shows the importance of reproducibility and control experiments to validate protein interactions detected in such settings. Finally it suggests that this strategy will not identify further CD4-binding proteins in the resting T lymphoid cells that were investigated here, as those interactions are probably of weak and/or transient nature.

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