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ALIX protein analysis: storage temperature may impair results

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ALIX [ALG-2 (apoptosis-linked gene 2)-interacting protein X] is one of the most well-known molecular biomarkers of extracellular vesicles. Extracellular vesicles are very small vesicles released by most cells and carry in their cargo components from the donor cells, thus being potent vehicles of intercellular (horizontal) communication, influencing various physiological and pathological functions of both recipient and donor cells. The increasing interest in extracellular vesicles highlights the key importance of a reliable analysis of this protein. However, several recent studies in the extracellular vesicles field have shown discrepancies in terms of the expression pattern of apoptosis-linked gene 2-interacting protein X upon Western blot analysis, differing from the theoretical expression pattern of apoptosis-linked gene 2-interacting protein X and its predicted molecular mass. Therefore, to address and clarify this point, we analyzed total protein cell lysates from a chronic myeloid leukemia cell line (K562) for the expression of apoptosis-linked gene 2-interacting protein X by Western blot and mass spectrometry analyses, using protein samples stored at different conditions regarding freezing temperature and storage time. We found that, when stored at -20 °C, a C-terminal specific proteolytic cleavage of apoptosis-linked gene 2-interacting protein X may occur, which depends on the length of storage time. We conclude that analysis of apoptosis-linked gene 2-interacting protein X protein expression should be only carried out when using a wide range of protease inhibitors during isolation of protein cell extract, while preferentially using fresh protein cell extracts or samples that were snap frozen in liquid nitrogen and stored at -80 °C. The current study highlights the importance of proper handling and storage of protein cell lysates for downstream applications in pre-clinical or clinical studies.

Keywords

ALIX; extracellular vesicles; protein degradation; reliable protein identification; storage temperature

1. Introduction

Extracellular vesicles (EVs) are very small particles (30-1000 nm) enclosed by a phospholipid bilayer, which do not replicate [1]. EVs are released by cells from a spectrum of organisms ranging from prokaryotes to higher eukaryotes and plants [2], and carry in their cargo contents from the donor cells, such as nucleic acids, proteins, lipids, and carbohydrates [3]. These vesicles may transfer their cargo to recipient cells, thus being important mediators of intercellular (horizontal) communication [4]. Importantly, EVs not only modulate physiological processes (e.g. tissue repair or embryonic development) but also pathological processes (e.g. cancer or autoimmune diseases) and may be found in biological fluids [5], thus being a potential source of biomarkers for various human diseases [6, 7].

The increasing interest in EVs provides great relevance to the proper analysis of ALIX, [ALG-2 (apoptosis-linked gene 2)-interacting protein X], since it has been defined as one of the well-established molecular markers of EVs [8, 9]. ALIX is an adaptor protein first described for its capacity to bind to the calcium-binding protein, ALG-2 [10, 11]. It is a ubiquitously expressed cytosolic protein that was initially identified due to its association with pro-apoptotic signaling components [12]. More recently,

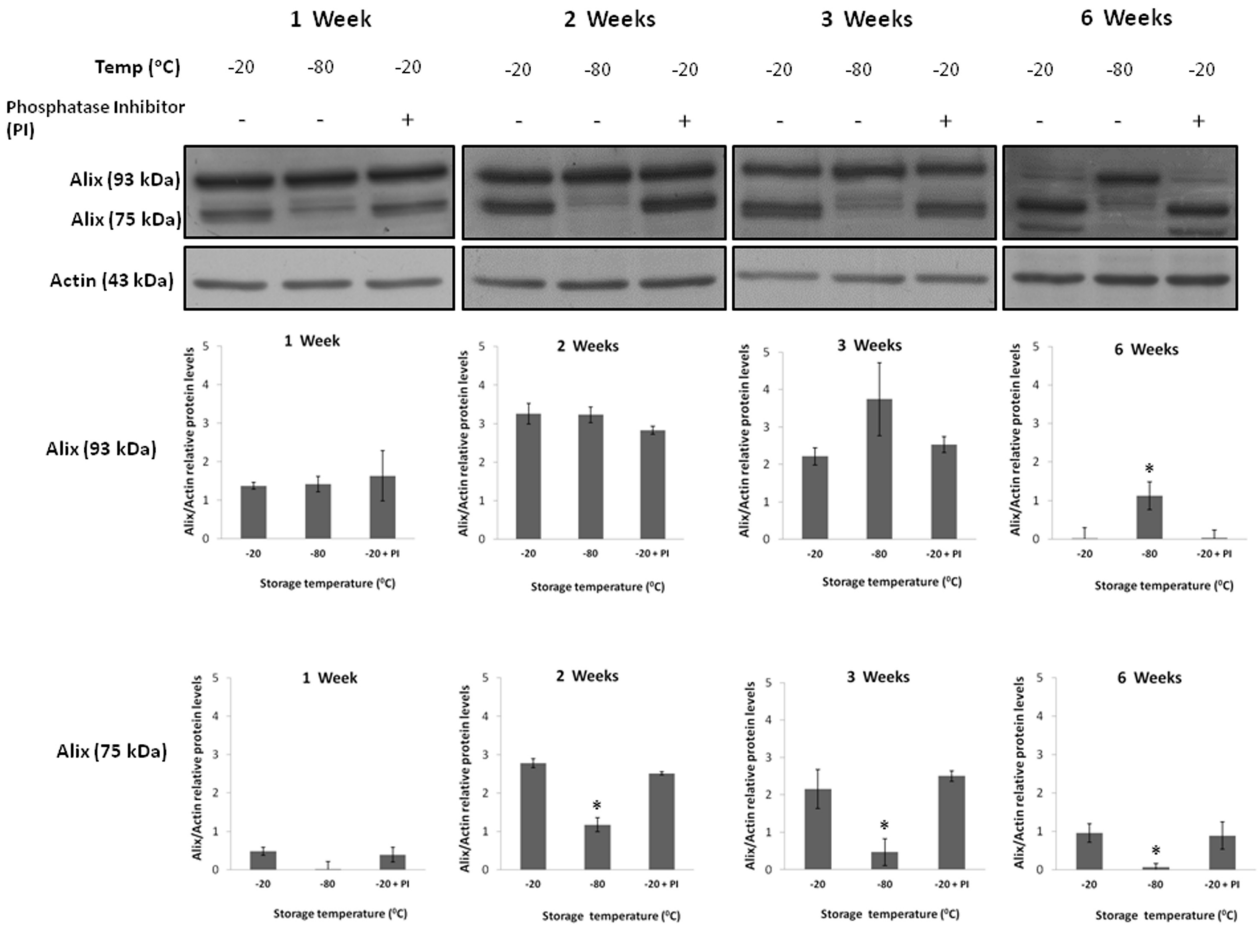

Figure 1. Western blot analysis of ALIX protein levels in K562 cells following various storage conditions.Fresh total cell protein lysates were immediately stored at: -20 °C , -20 °C with a phosphatase inhibitor cocktail (+ PI) or at -80 °C . Samples were kept frozen for 1, 2, 3 and 6 weeks. (A) Representative Western blot images; (B) Scanning densitometry analysis of the Western blot ALIX bands, represented as mean ± SEM from three in dependent experiments. β-Actin was used as a loading control. * $P \leq 0.05$ when comparing protein levels in samples stored at -20°C (-PI) vs. other storage conditions (-80 °C or -20 °C + PI).

ALIX was found to regulate other cellular mechanisms such as endocytic membrane trafficking [13], being essential for clathrin-independent endocytosis and signaling [11]. Much of the progress in characterizing the function of ALIX has been focused on its connection to endocytic membrane trafficking. This protein interacts with several ESCRT (endosomal sorting complexes required for transport) proteins in order to participate in the budding and abscission processes that can ultimately lead to the formation of exosomes [12, 14]. ALIX also increased protein content and protective functions of exo-somes shed by induced pluripotent stem cells [15]. ALIX is also associated with programmed cell death [16, 17], virus egress [18], cytokinesis [19], regulation of integrin-mediated cell adhesions and extracellular matrix assembly [20] and repair of the plasma membrane [21, 22].

The accession number of ALIX is Q8WUM4 and the UniProt ID is PDC6I_HUMAN. According to UniProt, the predicted molecular mass of the ALIX protein is 96.023 kDa, and upon Western blot analysis it is detected as a single band [4]. However, although some studies present only one band as expected [23], several reports identified more than a single ALIX band upon Western Blot analysis. Indeed, several ALIX-associated bands have been observed upon immunoblot analysis when using protein lysates derived from either cultured cell lines or EVs [24 - 29]. In some of these studies [24, 25, 27], ALIX appeared as two (or more) distinct bands, including a major one with the predicted molecular mass of approximately 93 kDa as well as additional bands with a smaller size. In other studies, although ALIX appeared as a single band with the expected molecular mass [26, 28, 29], the supplementary information (in which the respective uncropped blots are shown) revealed the presence of other band(s) which were not identified due to their surprising small molecular mass.

It is well known that a proper freezing storage temperature is one of the most important factors in order to maintain protein's integrity over an extended period of time as well as the use of a cocktail of protease inhibitors [30 - 33].Therefore, given the increased interest in ALIX in the last years [28, 34] and our own need to analyze the expression of this protein, the aim of the present study was to further characterize the molecular basis for the appearance of distinct bands of ALIX.

2. Materials and methods

2.1 Cell culture

The chronic myeloid leukemia cell line K562 was from European Collection of Authenticated Cell Cultures (ECACC). The cell line was genotyped and routinely monitored for mycoplasma contamination by PCR (VenorGeM® Advance Mycoplasma Detection Kit, Minerva). Cells were routinely grown in RPMI-1640 medium (with Ultraglutamine I and 25 mM HEPES pH 7.3; Lonza), supplemented with 10% fetal bovine serum (FBS, PAA) at 37 ºC in a humidified atmosphere of 5% CO_2. Cell numbers and viability were determined using the trypan blue exclusion assay. All experiments were carried out with exponentially growing cells having over 90% viability, as previously described [35, 36].

2.2 Analysis of ALIX protein expression upon Western Blots

Cells were harvested and washed with PBS and cell pellet was kept for different periods of 1, 2, 3 or 6 weeks at -20ºC or -80ºC. Following these variable storage times cell pellets were suspended in Winman's buffer (1% NP-40, 0.1 M Tris-HCl pH 8.0, 0.15 M NaCl, and 5 mM EDTA) with EDTA-free protease inhibitor cocktail (Catalogue number 11873580001, Roche), without phosphatase inhibitor (for samples kept at -20 ºC and at -80 ºC) or with phosphatase inhibitor (Sodium Orthovanadate, Catalogue number S6508, Sigma; for samples kept at -20 ºC). Protein was quantified using a modified Lowry assay (Bio-Rad). Western blot analysis was performed as previously described [35, 36]. Briefly, 20 μg of protein was resolved on a 10% Bis-Tris SDS-PAGE and transferred onto a nitrocellulose membrane (GE Healthcare). Membranes were then incubated with the following primary antibodies: anti-ALIX sc-49268 from Santa Cruz Biotechnology (1 : 100); anti-ALIX #2171 from Cell Signaling Technologies (1 : 100); anti-β-Actin from Santa Cruz Biotechnology (1 : 2000). The following secondary antibody was used: anti-goat IgG-HRP, diluted 1 : 2000, from Santa Cruz Biotechnology. Signal was detected using the ECL Western blot Detection Reagents (GE Healthcare), the Amersham Hyperfilm ECL (GE Healthcare), and the Kodak GBX developer and fixer (Sigma). Protein bands were evaluated in three independent experiments. The intensity of the bands obtained in each experiment was further analyzed using the software Quantity One-1D Analysis (Bio-Rad, USA). Two major bands of the ALIX protein were detected: a predicted one with a molecular mass of 93 kDa as well as a 75 kD species.

2.3 Protein identification analysis by mass spectrometry

Protein identification was performed by mass spectrometry using previously published procedures [37, 38]. Briefly, gel plugs were reduced with 25 mM DTT in 50 mM ammonium bicarbonate (ABC) for 20 min at 56 ºC and alkylated with 55 mM iodoacetamide in 50 mM ABC in the dark for 20 min at room temperature. In gel tryptic digestion was performed (20 ng trypsin per spot) at 37 ºC for 3h in the presence of surfactant at 0.01% in 50 mM ABC (Promega, Madison, WI, USA). Peptide extraction was performed using a 2.5% trifuoroacetate (TFA) solution. Protein digests were desalted and concentrated by C18 reverse phase chromatography (ZipTips, Millipore, Bedford, MA, USA) following the manufacturer's instructions. Samples were crystallized onto a stainless steel 384-well MALDI plate using the dried droplet method. For the matrix preparation, a solution of 7-8 mg/mL α-cyano-4-hydroxycinnamic acid in 50% acteonitrile/0.1% TFA was used. Samples were analyzed using a 4800 Plus MALDI TOF/TOF Analyzer (AB SCIEX, Framingham, MA, USA) as already described [39]. Peptide mass fingerprint (PMF) data were collected in positive MS reflector mode within the ion range at m/z 700-4000 and was calibrated internally using trypsin autolysis peaks. MS spectra were analyzed using the Mascot search engine software (Matrix Science, London, UK). The UniProt protein sequence database was considered for this analysis using the human reference proteome. Cysteine carbamidomethylation and methionine oxidation were considered as constant and variable modifications, respectively. Up to two missed trypsin cleavage sites were considered.

2.4 Statistical analysis

The statistical analysis of the scanning densitometry analysis of the Western blot was carried out using the Student's *t*-test. Data was analyzed with the paired test. Differences were considered statistically significant when $P \leq 0.05$, by comparing samples kept at -20 ºC without phosphatase inhibitor with samples kept at -20 ºC with phosphatase inhibitor or kept at -80 ºC without phosphatase inhibitor.

3. Results and discussion

Total protein cell lysates from a chronic myeloid leukemia cell line (K562, ECACC) were analyzed for ALIX protein expression by Western blot, using two of the most commonly employed antibodies (ALIX sc-49268 from Santa Cruz Biotechnology and ALIX #2171 from Cell Signaling Technologies). The samples were treated with an EDTA-free protease inhibitor cocktail to avoid protein degradation during storage at -20 ºC (with or without phosphatase inhibitors) or at -80 ºC for different periods of time (1, 2, 3 and 6 weeks) [35, 36].

After performing Western blot analysis of the stored protein lysates, we detected two major bands (using either antibody) as shown in Fig. 1: i) One 93 kDa band with a molecular mass compatible with the predicted size ALIX (93 kDa-ALIX) as well as ii) Another band with an approximate molecular mass of 75 kDa (75 kDa-ALIX).

In order to confirm that the antibodies were specifically detecting ALIX-based proteins, we performed mass spectrometry analysis (Supplementary Fig. S1 and 2). We detected 20 peptides related to ALIX using the 93 kDa protein band including an N-terminal peptide consisting of amino acids 24-41 as well as a C-terminal peptide encompassing amino acids 716-745. In the 75 kDa band, we still detected the N-terminal peptide, whereas the C- terminal 716-745 peptide was absent (Fig. 2). This led us to conclude that the 75 kDa protein band corresponds to a truncated form of ALIX in which the C-terminal peptide was missing presumably due to proteolysis.

It is important to note that for both the human and mouse ALIX proteins, there are published reports about the cathepsin-mediated proteolytic cleavage that may occur at their C-termini (the residue numbers are based on Uniprot sequences) as follows: a) Regarding the human ALIX protein, there is a report based on proteomics screen for cathepsins which reveals a putative cleavage site by cathepsin L between amino acid residues A732 and G733; this occurred within the sequence QSSPA^GGHAPT (with ^ representing the putative cleavage site) where the human ALIX appears

Database: UniProtKB.2013.6.17
Accession Number: Q8WUM4
Uniprot ID: PDC6I_HUMAN
Species: HUMAN
Name: Programmed cell death 6-interacting protein
Organism: Homo sapiens
Gene: PDCD6IP

A – Sequence Alix 93 kDa band

1 MATFISVQLK KTSEVDLAKP LVKFIQQTYP SGGEEQAQYC RAAEELSKLR RAAVGRPLDK HEGALETLLR YYDQICSIEP
81 KFPFSENQIC LTFTWKDAFD KGSLFGGSVK LALASLGYEK SCVLFNCAAL ASQIAAEQNL DNDEGLKIAA KHYQFASGAF
161 LHIKETVLSA LSREPTVDIS PDTVGTLSLI MLAQAQEVFF LKATRDKMKD AIIAKLANQA ADYFGDAFKQ CQYKDTLPKE
241 VFPVLAAKHC IMQANAEYHQ SILAKQQKKF GEEIARLQHA AELIKTVASR YDEYVNVKDF SDKINRALAA AKKDNDFIYH
321 DRVPDLKDLD PIGKATLVKS TPVNVPISQK FTDLFEKMVP VSVQQSLAAY NQRKADLVNR SIAQMREATT LANGVLASLN
401 LPAAIEDVSG DTVPQSILTK SRSVIEQGGI QTVDQLIKEL PELLQRNREI LDESLRLLDE EEATDNDLRA KFKERWQRTP
481 SNELYKPLRA EGTNFRTVLD KAVQADGQVK ECYQSHRDTI VLLCKPEPEL NAAIPSANPA KTMQGSEVVN VLKSLLSNLD
561 EVKKEREGLE NDLKSVNFDM TSKFLTALAQ DGVINEEALS VTELDRVYGG LTTKVQESLK KQEGLLKNIQ VSHQEFSKMK
641 QSNNEANLRE EVLKNLATAY DNFVELVANL KEGTKFYNEL TEILVRFQNK CSDIVFARKT ERDELLKDLQ QSIAREPSAP
721 SIPTPAYQSS PAGGHAPTPP TPAPRTMPPT KPQPPARPPP PVLPANRAPS ATAPSPVGAG TAAPAPSQTP GSAPPPQAQG
801 PPYPTYPGYP GYCQMPMPMG YNPYAYGQYN MPYPPVYHQS PGQAPYPGPQ QPSYPFPQPP QQSYYPQQ

B – Sequence Alix 75 kDa band

1 MATFISVQLK KTSEVDLAKP LVKFIQQTYP SGGEEQAQYC RAAEELSKLR RAAVGRPLDK HEGALETLLR YYDQICSIEP
81 KFPFSENQIC LTFTWKDAFD KGSLFGGSVK LALASLGYEK SCVLFNCAAL ASQIAAEQNL DNDEGLKIAA KHYQFASGAF
161 LHIKETVLSA LSREPTVDIS PDTVGTLSLI MLAQAQEVFF LKATRDKMKD AIIAKLANQA ADYFGDAFKQ CQYKDTLPKE
241 VFPVLAAKHC IMQANAEYHQ SILAKQQKKF GEEIARLQHA AELIKTVASR YDEYVNVKDF SDKINRALAA AKKDNDFIYH
321 DRVPDLKDLD PIGKATLVKS TPVNVPISQK FTDLFEKMVP VSVQQSLAAY NQRKADLVNR SIAQMREATT LANGVLASLN
401 LPAAIEDVSG DTVPQSILTK SRSVIEQGGI QTVDQLIKEL PELLQRNREI LDESLRLLDE EEATDNDLRA KFKERWQRTP
481 SNELYKPLRA EGTNFRTVLD KAVQADGQVK ECYQSHRDTI VLLCKPEPEL NAAIPSANPA KTMQGSEVVN VLKSLLSNLD
561 EVKKEREGLE NDLKSVNFDM TSKFLTALAQ DGVINEEALS VTELDRVYGG LTTKVQESLK KQEGLLKNIQ VSHQEFSKMK
641 QSNNEANLRE EVLKNLATAY DNFVELVANL KEGTKFYNEL TEILVRFQNK CSDIVFARKT ERDELLKDLQ QSIAREPSAP
721 SIPTPAYQSS PAGGHAPTPP TPAPRTMPPT KPQPPARPPP PVLPANRAPS ATAPSPVGAG TAAPAPSQTP GSAPPPQAQG
801 PPYPTYPGYP GYCQMPMPMG YNPYAYGQYN MPYPPVYHQS PGQAPYPGPQ QPSYPFPQPP QQSYYPQQ

Figure 2. Mass spectrometry analysis of the two ALIX bands. The sequences of both the 93 kDa (A) and 75 kDa (B) bands were analyzed and the peptides related to ALIX were detected. The C-terminal 716-745 peptide, which was present in the sequence of the intact 93 kDa protein (A, marked with a box) was absent from the sequence of the 75 kDa protein (B).

under protein IPI ID IPI00246058.7 [40]. Whereas, for the mouse ALIX protein, there is a previous paper also based on a proteomics screen that cathepsin E cleaved the murine ALIX protein between residues L705 and L706, within the sequence ERDEL^LKDLQ [41]. Thus, proteomics analysis as well as protease cleavage analysis reveals that the C-terminal ends of both the human and murine ALIX proteins are subject for proteolytic cleavage mediated by cathepsins. Thus, these C-terminal truncations of both the human and mouse ALIX proteins, are in accord with the difference observed between the native 93 kDa protein and the truncated 75 kDa protein in our present study.

When analyzing the protein cell extracts stored at -20 ºC over time (1, 2, 3 and 6 weeks), both in the presence or absence of phosphatase inhibitors, we noticed that the presumed C-terminal proteolytic degradation of ALIX depended on the duration of the storage of samples at -20 ºC (Fig. 1). In fact, over a prolonged storage time and at -20 ºC , the levels of the primary 93 kDa ALIX protein decreased, while the levels of the truncated form (75 kDa-ALIX) increased. In addition, under the same conditions (-20 ºC storage), the presence of a phosphatase inhibitor had no effect on the level of the degradation of this protein (Fig. 1).

In contrast, when analyzing the samples stored at -80 ºC for the same period of time (1, 2, 3 and 6 weeks), no truncation of ALIX was observed. In fact, the level of the 93 kDa ALIX protein remained unaltered during the 6 weeks period and no increase in the truncated form of ALIX (75 kDa-ALIX) was observed (Fig. 1).

Long-term storage of protein lysates at -80 ºC is recommended [30], but -20 ºC is commonly used for shorter storage periods. Therefore, the degradation observed in ALIX after a short storage period (1 or 2 weeks) at -20 ºC was rather unexpected. Of note, Western blot analysis was carried out at different times by two of the authors (using the same experimental conditions), which eliminates errors due to incorrect sample processing and analysis.

The obtained results demonstrate that analysis of ALIX protein upon immunoblot analysis should be carried out when using freshly isolated protein extracts or that have been properly stored at -80 ºC with the use of proper protease inhibitors, even for a short storage time. This would avoid misleading conclusions resulting from the analysis of decreased ALIX protein levels due to degradation.

This study emphasizes the importance of proper isolation, handling and storage of protein cell lysates for downstream applications such as Western blots, when quantifying ALIX protein levels.

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Conflict of interest

The authors declare no competing interests.

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[–] Parameters

Database: **UniProtKB.2013.6.17**
Accession Number: **Q8WUM4**
Considered modifications: | **Oxidation (M)** |
Digest Used: **Trypsin**
Max. # Missed Cleavages: **2**
User AA Formula 1: **C2 H3 N1 O1**
Constant Modification: **Carbamidomethyl (C)**
Minimum Digest Fragment Mass: **500**
Maximum Digest Fragment Mass: **4000**
Minimum Digest Fragment Length: **5**

Acc. #: Q8WUM4 **Uniprot ID:** PDC6I_HUMAN **Species:** HUMAN **Name:** Programmed cell death 6-interacting protein
Organism: Homo sapiens **Gene:** PDCD6IP **Existence:** Evidence at protein level **Version:** 1
Index Number: **284357**
pI of Protein: **6.1**
Protein MW: **96024**
Amino Acid Composition: **A89 C11 D44 E59 F30 G39 H11 I35 K63 L83 M14 N37 P73 Q60 R34 S56 T45 V50 W2 Y33**

```
1   MATFISVQLK KTSEVDLAKP LVKFIQQTYP SGGEEQAQYC RAAEELSKLR RAAVGRPLDK HEGALETLLR YYDQICSIEP
81  KFPFSENQIC LTFTWKDAFD KGSLFGGSVK LALASLGYEK SCVLFNCAAL ASQIAAEQNL DNDEGLKIAA KHVQFASGAF
161 LHIKETVLSA LSREPTVDIS PDTWGTLSLI MLAQAQEVFF LKATRDKMKD AIIAKLANQA ADYFGDAFKQ CQYKDTLPKE
241 VFPVLAAKHC IMQANAEYHQ SILAKQQKKF GEEIARLQHA AELIKTVASR YDEYVNVKDF SDKINRALAA AKKDNDFIYH
321 DRVPDLKDLD PIGKATLVKS TPVNVPISQK FTDLFEKMVP VSVQQSLAAY NQRKADLVNR SIAQMREATT LANGVLASLN
401 LPAAIEDVSG DTVPQSILTK SRSVIEQGGI QTVDQLIKEL PELLQENREI LDESLRLLDE EEATDNDLRA KFKERWQRTP
481 SNELYKPLRA EGTNFRTVLD KAVQADGQVK ECYQSHRDTI VLLCKPEPEL NAAIPSANPA KTMQSSEVVN VLKSLLSNLD
561 EVKKEREGLE NDLKSVNFDM TSKFLTALAQ DGVINEEALS VTELDEVYGG LTTKVQESLK KQEGLLKNIQ VSHQEFSKMK
641 QSNNEANLRE EVLKNLATAY DNFVELVANL KEGTKFYNEL TEILVRFQNK CSDIVFARKT ERDELLKDLQ QSIAREPSAP
721 SIPTPAYQSS PAGGHAPTPP TPAPRTMPPT KPQPPARPPP PVLPANRAPS ATAPSPVGAG TAAPAPSQTP GSAPPPQAQG
801 PPVPTYPGYP GYCQMPMPMG YNPYAYGQYN MPYPPVYHQS PGQAPYPGPQ QFSYPPQPP QQSYYPQQ
```

The matched peptides cover **31%** (268/868 AA's) of the protein.

m/z Submitted	MH+ Matched	Intensity	Delta ppm	Modifications	Start	End	Missed Cleavages	Sequence
949.5155	949.5102	25.0	5.63		269	276	1	(K)KFGEEIAR(L)
997.5702	997.5677	59.0	2.58		439	446	0	(K)ELPELLQR(N)
1172.6616	1172.6746	31.0	-11.1		42	51	2	(R)AAEELSKLRR(A)
1244.6528	1244.6593	31.0	-5.23		447	456	1	(R)NREILDESLR(L)
1316.6599	1316.6593	34.0	0.433		628	638	0	(K)NIQVSHQEFSK(M)
1330.6830	1330.6961	31.0	-9.87		564	574	2	(K)KEREGLENDLK(S)
1396.7234	1396.7471	63.0	-17.0		676	686	0	(K)FYNELTEILVR(F)
1501.8368	1501.8268	37.0	6.68		374	386	2	(R)KADLVNRSIAQMR(E)
1790.9106	1790.9218	56.0	-6.22		358	373	0	(K)MVPVSVQQSLAAYNQR(K)
1919.0062	1919.0167	72.0	-5.48		358	374	1	(K)MVPVSVQQSLAAYNQRK(A)
2007.0151	2007.0029	25.0	6.09		457	473	2	(R)LLDEEEATDNDLRAKFK(E)
2093.0879	2093.0774	42.0	5.00		479	496	1	(R)TPSNELYKPLRAEGTNFR(T)
2113.1050	2113.1400	47.0	-16.6		621	638	2	(K)KQEGLLKNIQVSHQEFSK(M)
2161.9602	2161.9607	55.0	-0.247		24	41	0	(K)FIQQTYPSGGEEQAQYC(Carbamidomethyl)R(A)
2504.2932	2504.2879	26.0	2.13		584	606	0	(K)FLTALAQDGVINEEALSVTELDR(V)
2542.3005	2542.2653	27.0	13.9	1Oxidation	249	269	2	(K)HC(Carbamidomethyl)IMQANAEYHQSILAKQQKK(F)
2936.5037	2936.4537	64.0	17.0		716	745	0	(R)EPSAPSIPTPAYQSSPAGGHAPTPPTPAPR(T)
3162.6523	3162.6643	30.0	-3.78		174	202	0	(R)EPTVDISPDTVGTLSLIMLAQAQEVFFLK(A)
3178.6352	3178.6592	67.0	-7.53	1Oxidation	174	202	0	(R)EPTVDISPDTVGTLSLIMLAQAQEVFFLK(A)
3323.8000	3323.7369	69.0	19.0		584	614	1	(K)FLTALAQDGVINEEALSVTELDRVYGGLTTK(V)

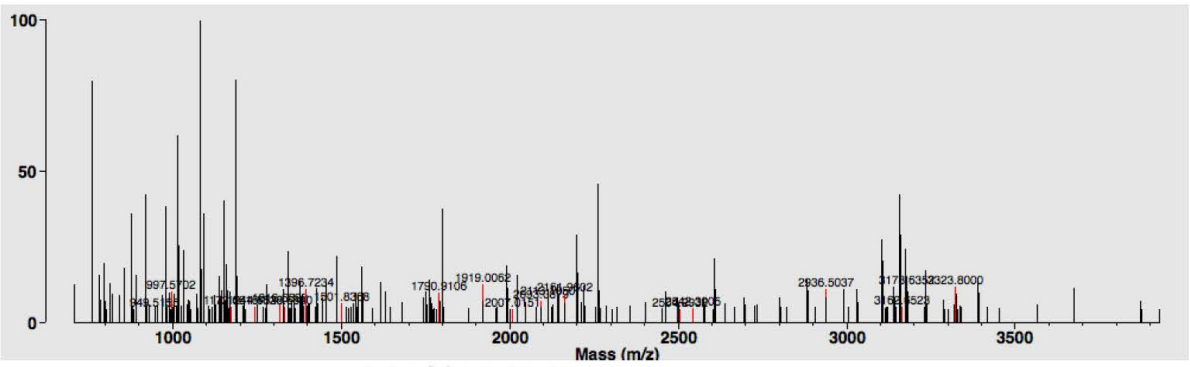

Figure S1. Mass spectrometry analysis of the 93 kDa ALIX band. The analysis of the sequence of the 93 kDa protein is shown in detail (sequences and peaks of the peptides detected).

[–] **Parameters**

Database: **UniProtKB.2013.6.17**
Accession Number: **Q8WUM4**
Considered modifications: | **Oxidation (M)** |
Digest Used: **Trypsin**
Max. # Missed Cleavages: **2**
User AA Formula 1: **C2 H3 N1 O1**
Constant Modification: **Carbamidomethyl (C)**
Minimum Digest Fragment Mass: **500**
Maximum Digest Fragment Mass: **4000**
Minimum Digest Fragment Length: **5**

Acc. #: Q8WUM4 **Uniprot ID:** PDC6I_HUMAN **Species:** HUMAN **Name:** Programmed cell death 6-interacting protein
Organism: Homo sapiens **Gene:** PDCD6IP **Existence:** Evidence at protein level **Version:** 1
Index Number: **284357**
pI of Protein: **6.1**
Protein MW: **96024**
Amino Acid Composition: **A89 C11 D44 E59 F30 G39 H11 I35 K63 L83 M14 N37 P73 Q60 R34 S56 T45 V50 W2 Y33**

```
1   MATFISVQLK KTSEVDLAKF LVKFIQQTYP SGGEEQAQYC RAAEELSKLR RAAVGRPLDK HEGALETLLR YYDQICSIEP
81  KFPFSENQIC LTFTWKDAFD KGSLFGGSVK LALASLGYEK SCVLFNCAAL ASQIAAEQNL DNDBGLKIAA KHYQFASGAF
161 LHIKETVLSA LSRRPTVDIS PDTVGTLSLI MLAQAQEVFF LKATRDKMKD AIIAKLANQA ADYPGDAPKQ CQVKDTLPKE
241 VFPVLAAKHC IMQANAEYHQ SILAKQQKKF GEEIARLQHA AELIKTVASR YDEYVNVKDF SDKINRALAA AKKDNDFIYH
321 DRVPDLKDLD PIGKATLVKS TPVHVPISQK FTDLFEKMVP VSVQQSLAAY NQRKADLVNR SIAQMREATT LANGVLASLN
401 LPAAIEDVSG DTVPQSILTK SRSVIEQGGI QTVDQLIKEL PELLQRNREI LDESLRLLDE EEATDNDLRA KFKERWGRTP
481 SNELYKPLRA EGTNFRTVLD KAVQADGQVK ECYQSHRDTI VLLCKPEPEL NAAIPSANPA KTMQGSEVVN VLKSLLSNLD
561 EVKKEREGLE NDLKSVNFDM TSFFLTALAQ DGVINEEALS VTELDRVYGG LTTKVQESLK KQEGLLKNIQ VSHQEFSKMK
641 QSNNEANLRE EVLKNLATAY DNFVELVANL KEGTKFYNEL TEILVRFQNK CSDIVFARKT ERDELLKDLQ QSIAREPSAP
721 SIPTPAYQSS PAGGHAPTPP TPAPETMPPT KPQPPARPPP PVLPANRAPS ATAPSPVGAG TAAPAPSQTP GSAPPPQAQG
801 PFYPTYPGYP GYCQMPMPMG YNPYAYGQYN MPYPPVYHQS PGQAPYPGPQ QPSYPFPQPP QQSYYPQQ
```

The matched peptides cover **22%** (193/868 AA's) of the protein.

m/z Submitted	MH+ Matched	Intensity	Delta ppm	Modifications	Start	End	Missed Cleavages	Sequence
815.4987	815.4985	36.0	0.160		621	627	1	(K)KQEGLLK(N)
821.4307	821.4152	29.0	18.9		270	276	0	(K)FGEEIAR(L)
997.5497	997.5677	65.0	-18.0		439	446	0	(K)ELPELLQR(N)
1244.6536	1244.6593	28.0	-4.64		447	456	1	(R)NREILDESLR(L)
1317.6902	1317.7161	27.0	-19.7		479	489	0	(R)TPSNELYKPLR(A)
1389.7302	1389.7267	28.0	2.53	1Oxidation	375	386	1	(K)ADLVNRSIAQMR(E)
1396.7391	1396.7471	54.0	-5.69		676	686	0	(K)FYNELTEILVR(F)
1427.7096	1427.7165	27.0	-4.85		97	110	1	(K)DAFDKGSLFGGSVK(L)
1643.8251	1643.8347	28.0	-5.88		641	654	1	(K)QSNNEANLREEVLK(N)
1790.9235	1790.9218	51.0	0.938		358	373	0	(K)MVPVSVQQSLAAYNQR(K)
1935.0160	1935.0117	104.0	2.24	1Oxidation	358	374	1	(K)MVPVSVQQSLAAYNQRK(A)
2004.9628	2004.9661	29.0	-1.68		291	306	2	(R)YDEYVNVKDFSDKINR(A)
2093.0718	2093.0774	46.0	-2.70		479	496	1	(R)TPSNELYKPLRAEGTNFR(T)
2161.9663	2161.9607	71.0	2.57		24	41	0	(K)FIQQTYPSGGEEQAQYC(Carbamidomethyl)R(A)
2504.2993	2504.2879	61.0	4.57		584	606	0	(K)FLTALAQDGVINEEALSVTELDR(V)
3822.0200	3821.9895	29.0	7.99		340	373	2	(K)STPVNVPISQKFTDLFEKMVPVSVQQSLAAYNQR(K)

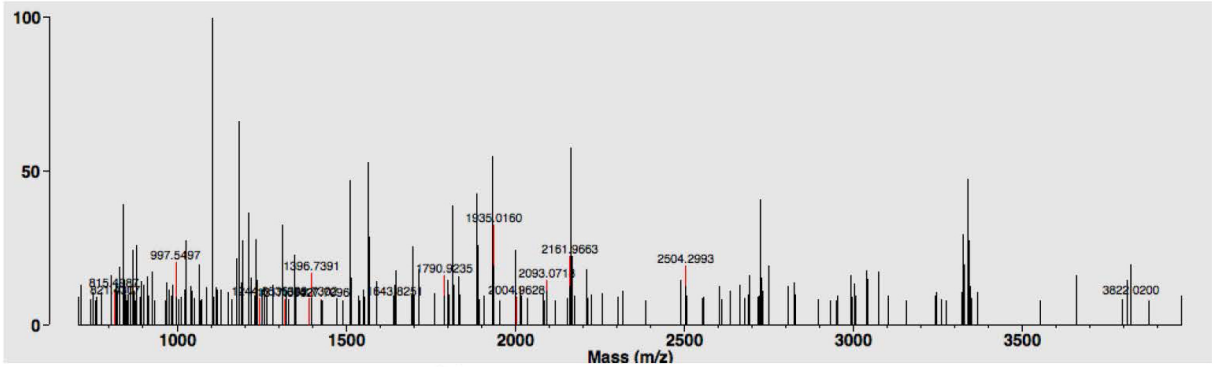

Figure S2. Mass spectrometry analysis of the 75 kDa ALIX band. The analysis of the sequence of 75 kDa protein is shown in detail (sequences and peaks of the peptides detected).