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Eight Full-Length Abelson Related Gene (Arg) Isoforms Are Constitutively Expressed in Caki-1 Cell Line and Cell Distribution of Two Isoforms Has Been Analyzed After Transfection

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ABSTRACT

The human Arg (Abl2) nonreceptor tyrosine kinase has a role in cytoskeletal rearrangements by its C-terminal F-actin- and microtubule-binding sequences. We have previously identified Arg transcripts with different 5′- and 3′-ends, named respectively long and short 1A and 1B (1AL, 1AS, 1BL, 1BS) and long and short C-termini (CTL and CTS), that have different expression patterns in various cell types. The combination of the different ends permits to predict eight putative full-length Arg transcripts and corresponding proteins. By Reverse Transcription-Long PCR we show here that all eight full-length transcripts are endogenously expressed in Caki-1 cells and the two bands, ≃10 kDa different, shown by 1-D Western blots of Hek293T and Caki-1 lysates correspond to the full-length Arg protein isoforms with different C-termini. 2-D Western blot analysis evidenced different high molecular weight and slight acidic specific spots in Hek293T and Caki-1 lysates. The cellular localization of two Arg isoforms (1BLCTL and 1BLCTS) transfected in Caki-1 and Hek293T cells was cytoplasmic, and some differences in cytoskeleton interactions have been evidenced. Moreover, in Hek293T cells only the transfected 1BLCTS isoform gives rise to a large intracytoplasmic cylindrical structure containing phalloidin-positive amorphous actin aggregates. The presence of eight full-length Arg isoforms with different cellular expression may imply a diverse functional role in normal and neoplastic cells. J. Cell. Biochem. 105: 1219–1227, 2008.

KEY WORDS: ARG TYROSINE KINASE; FULL-LENGTH ISOFORMS; ENDOGENOUS EXPRESSION; CELLULAR TRANSFECTION; SUBCELLULAR DISTRIBUTION

Arg (also known as Abl2) is a member of the Abelson family of nonreceptor tyrosine protein kinase [Kruh et al., 1990; Pendergast, 2002] showing a high degree of amino acid sequence identity with c-Abl in the tyrosine kinase, SH2 and SH3 domains and also the presence of an alternative 1A and 1B first exon [Kruh et al., 1990]. The long C-terminal domain of Arg contains three proline-rich sequences that bind to the SH3 domains of adaptor proteins [Mysliwiec et al., 1996] and two F-actin- and one microtubule-binding sequences [Pendergast, 2002]. Human Arg is ubiquitously expressed with highest levels in nervous tissue [Perego et al., 1991]. Arg mRNA increases during granulocytic and macrophage-like differentiation of HL-60 cells [Perego et al., 1998] and is more abundant in mature than in immature B lymphoid cell lines [Bianchi et al., 2002]. Arg is involved in human neoplastic diseases, in fact ARG gene is translocated with ETV6 gene in human acute leukemia [Cazzaniga et al., 1999; Iijima et al., 2000; Griesinger et al., 2002] and is up- or down-regulated in several solid tumors [Lu et al., 1997; Chen et al., 1999; Crnogorac-Jurcevic et al., 2002; Liu et al., 2002; Shen et al., 2003; Sasaki et al., 2004; Srinivasan and Plattner, 2006]. Through its interactions with cytoskeletal structures Arg plays redundant actions with c-Abl in murine neurulation [Koleske et al., 1998], is required for adhesion-dependent neurite branching [Moresco et al., 2005] and synapse/dendrite stability [Sfakianos et al., 2007]. Moreover, Arg has a role in fibroblastic- and epithelial-cell adhesion and migration [Hernandez et al., 2004; Hu et al., 2005], in particular Arg inhibits fibroblast migration by attenuating actomyosin contractility and regulating focal adhesion dynamics [Peacock et al., 2007]. We have previously identified different

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5′- and 3′-ends of Arg transcripts [Perego et al., 2005a] in human cells, in addition to those initially described [Kruh et al., 1990]. There are four different 5′-ends, named long and short 1A (1AL and 1AS) and long and short 1B (1BL and 1BS), because of the alternative splicing of exon II. There are also two different 3′-ends, named long and short C-termini (CTL and CTS), because of alternative absence of a sequence including part of the most 5′ (internal) F-actin binding domain. These ends are differently expressed in normal and tumor cells, and during cell differentiation [Perego et al., 2005a]. The combination of the different 5′- and 3′-splicing events described made possible to predict eight full-length Arg transcripts and corresponding proteins.

In this work, we wanted to verify whether all the predicted full-length transcripts are endogenously expressed in Caki-1 cells and to identify the corresponding protein isoforms. In addition, we wanted to characterize the cellular distribution of some of these isoforms.

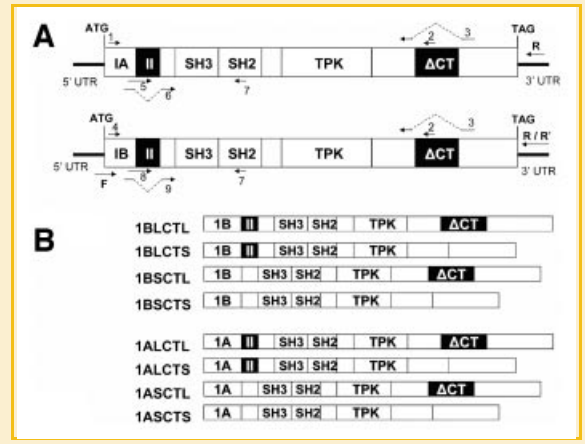

Fig. 1. Schematic representation of ARG gene products. A: Localizations, indicated by arrows, of the specific reverse primer R used for cDNA synthesis, the forward and reverse primers used in the first round and in the nested PCR assay and the specific forward F and reverse R′ primers used for cloning. B: The eight full-length Arg isoforms predicted by the combination of the different splicing events at the 5′- and 3′-ends. The alternative spliced exon II (II) and the C-terminal region (CT), lacking in the CTS forms, are shown in black. The first alternative exons 1A and 1B, the SH3, SH2 and tyrosine protein kinase (TPK) domains are also indicated.

MATERIALS AND METHODS

CELL LINES

The Caki-1 clear cell renal carcinoma cell line and Hek293T human embryonic kidney cell line, from American Type Culture Collection, were cultured in DMEM with 10% FCS and tested during exponential growth.

RNA EXTRACTION AND CDNA SYNTHESIS

Total RNA was obtained by TRIZOL extraction (Invitrogen, Carlsbad, CA) and treated by DNAse; RNA was spectrophotometrically quantified and analyzed on 1% agarose gel. RNA yield per cell was obtained by calculating total cell number in a Thoma chamber and cell mortality was assessed by trypan blue dye. For Real-Time PCR, 8 g of RNA were reverse transcribed in a 40 l reaction in presence of 0.5 g of random examers [Perego et al., 1998]. For Reverse Transcription-Long PCR 4 g of RNA were reverse transcribed in a 20 l reaction in presence of 20 pmol of the specific reverse primer R (5′-AACAAGTCCTTTTCCCTCTCCC-3′) recognizing a sequence in the 3′-end region just downstream to Arg stop codon (see Fig. 1A).

QUANTITATIVE REAL-TIME PCR

Real-Time PCR with TaqMan chemistry, primers and probes were used as previously described [Perego et al., 2005a]. For each cell line we assayed three different cDNA in duplicate. The transcript of GAPDH gene was amplified as an endogenous control of RNA quality. The relative amount of different 5′- and 3′-ends of Arg transcripts was calculated and expressed as $2^{-\Delta CT}$ [Perego et al., 2005a]. For absolute quantification of total Arg transcript, Real-Time PCR amplifications were carried out using 1 l of reverse transcription reaction product, corresponding to 200 ng of cDNA. Primers and probe used were those for total Arg amplification [Perego et al., 2005a]. The calibration curve was obtained with serial 10-fold dilutions, from 1×10^7 to 10 molecules of pFLAG-CMV2 plasmid containing Arg 1BLCTL isoform, each assayed in triplicate. Linear amplification down to the last dilution point was obtained (correlation coefficient 0.996, slope 3.32). All the data were analyzed using the SDS 2.1 software (Applied Biosystems) to interpolate the plasmid calibration curve data with amplification cycle threshold (Ct) of the unknown target sample, thus obtaining the number of Arg transcript molecules per g of RNA in the experimental sample.

ANALYSIS BY REVERSE TRANSCRIPTION-LONG PCR AND NESTED PCR

One microliter of cDNA was amplified in presence of 0.4 M primers, 0.5 mM dNTPs, DMSO 2%, 2.5 U AccuTaq LA DNA polymerase and 1× manufacturer's buffer (Sigma-Aldrich, St. Louis, MO) in a 50 l reaction. The amplification program was 98°C/30 s, (94°C/15 s; 60°C/20 s; 68°C/4 min) × 40 cycles and 68°C/10 min. Five microliters of amplification reaction products were run on 1% agarose gel. For amplifying the full-length isoforms the primers used in this first round PCR were: 1 and 2 that raise the expected amplicons of 2,054 and 1,991 bp (corresponding to 1ALCTL and 1ASCTL isoforms respectively); 1 and 3 with expected amplicons of 1,994 and 1,931 bp (1ALCTS and 1ASCTS isoforms); 4 and 2 with expected amplicons of 2,110 and 2,047 bp (1BLCTL and 1BSCTL isoforms); 4 and 3 with expected amplicons of 2,037 and 1,974 bp (1BLCTS and 1BSCTS isoforms). One microliter of the first amplification products were amplified in a second round of amplification (nested PCR assay) in presence of 0.4 M primers, 0.2 mM dNTPs, 2 mM $MgCl_2$, 2.5 U AmpliTaq Gold polymerase and 1× manufacturer's buffer (Applied Byosystems, Foster City, CA) in a 50 l reaction. The nested amplification program was 95°C/10 min, (94°C/30 s; 60°C/30 s; 72°C/30 s) × 40 cycles and 72°C/10 min. Ten microliters of these amplification reaction products were run on 1.8% agarose gel. In the nested amplification primers 5 and 7 raised an amplicon of 400 bp that revealed the presence of 1ALCTL and 1ALCTS isoforms, primers 6 and 7 raised amplicon of 320 bp revealing the presence of 1ASCTL and 1ASCTS isoforms, primers

8 and 7 raised amplicon of 398 bp revealing the presence of 1BLCTL and 1BLCTS isoforms, primers 9 and 7 raised amplicon of 339 bp revealing the presence of 1BSCTL and 1BSCTS isoforms.

Standard procedures were used to avoid false positive results. The primers used in the combinations described (see also Figs. 1A and 4) were tested for specificity and had the following sequences and localizations:

primer 1: 5′-GGCAGAGATCAGGACACT-3′ forward (exon IA)
primer 2: 5′-ACCAGATTCGCCTCTTGCTG-3′ reverse (exon XII)
primer 3: 5′-CTGCTCTGGAAGCCccgtg-3′ reverse (exon XII)
primer 4: 5′-AAGCTCCGGGGCTCCAGC-3′ forward (exon IB)
primer 5: 5′-TCTGCTCTACCCGACTTAACAGatc-3′ forward (exon 1A–II)
primer 6: 5′-TGCTCTACCCGACTTAACAGaagc-3′ forward (exon 1A–III)
primer 7: 5′-ACACAGGTCCATGGTACC-3′ reverse (exon IV)
primer 8: 5′-ATATCTTCACCCAGCATGatcac-3′ forward (exon 1B–II)
primer 9: 5′-CAATATCTTCACCCAGCATGaag-3′ forward (exon 1B–III)

The capital and lower case letters of primers 3, 6, 9 show the fusion point of the sequences that juxtapose after the loss of CT fragment and exon II respectively, and for primers 5 and 8 the border with exon II.

1-D AND 2-D WESTERN BLOTTING

Sixty micrograms of proteins from cell lysates obtained from transfected and untransfected Hek293T and Caki-1 cells and quantified with a Bio-Rad microassay (Hercules, CA), were separated in 7% polyacrylamide gel electrophoresis [Perego et al., 2005a]. Proteins (0.5 mg) obtained from untransfected cells [Perego et al., 2005b], or 2.5 l of 2-D SDS–PAGE Standards (Bio-Rad) were loaded onto 7 cm 3–10 NL IPG-strips (Amersham Pharmacia Biotech, Uppsala, Sweden) and then separated in 7% polyacrylamide gel electrophoresis. 1-D and 2-D gels were blotted onto PVDF membranes [Perego et al., 2005b] and blots were stained with Amido Black, to check transferred proteins. 2-D blots were also scanned through Image Scanner (Amersham Pharmacia Biotech). After blocking, 1-D blots were incubated overnight at 4°C with rabbit polyclonal anti-Arg antibodies directed against SH2 and SH3 domains (dilution 1:400; Upstate, Temecula, CA), mouse monoclonal anti-Arg antibodies directed against a C-terminal region corresponding to amino acids 743–842 (dilution 1:250; Abnova, Tapei City, Taiwan), mouse monoclonal anti-FLAG antibodies (M2, dilution 1:1000; Sigma-Aldrich) and anti--actin antibodies (dilution 1:1500; Sigma-Aldrich). 2-D blots were incubated with rabbit polyclonal anti-Arg antibodies (dilution 1:400; Upstate). The detection was performed with secondary antibodies coupled with horseradish peroxidase for 1 h at room temperature and SuperSignal West Femto/Pico Detection System (Pierce, Rockford, IL). The molecular weight and isoelectric point of proteins detected by 2-D Western blots was obtained by matching the autoradiographies to corresponding Amido Black stained membranes and to membrane with standards. Image Scanner and Melanie 4 software (GeneBio, Geneva, Switzerland) were used for matching.

VECTORS, TRANSFECTIONS AND IMMUNOFLUORESCENCE MICROSCOPY

Full-length Arg 1BLCTL cDNA, obtained by reverse transcription of Hek293T RNA with specific primer R′(5′-GTCGACAACAA-CAAGTCCTTTTCCCTCTCCC-3′ reverse) and by amplification with Long PCR using primers R' and F (5′-GTCGACGCAGGGATGGGG-CAGCAGCA-3′ forward) (see Fig. 1A), were cloned into the Sal I site of pFLAG-CMV2 vector (Sigma-Aldrich). Bacterial clones transformed by this construct were identified by restriction analysis and sequencing. The deletion of 309 bp CT sequence from 1BLCTL insert, corresponding to amino acids 688–790 [Perego et al., 2005a], was performed with QuickChange Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA) in order to obtain the Arg 1BLCTS cDNA cloned in pFLAG-CMV2. The following mutagenic primers were used: 5′-CCATAAGAAATACGAACTCACGGGGCTTCCAGAGCAGG ATAG-3′(forward) and 5′-GCCATCCTATCCTGCTCTGGAAGCCCC-GTGAGTTCGTATTTC-3′(reverse). The deletion was confirmed by sequencing.

Transient transfections of Caki-1 and Hek293T cell lines were performed with Arrest-in Transfection Reagent (OPEN Biosystems, Huntsville, AL) according to the manufacturer's specification. Cells grown on glass coverslips were fixed in absolute methanol 24 h after transfection and stained with mouse monoclonal FITC-conjugated anti-FLAG antibodies (dilution 1:50, Sigma-Aldrich) and mouse monoclonal anti- tubulin antibodies (dilution 1:20; Molecular Probes, Invitrogen, Carlsberg, CA) for 2 h at room temperature, and with Alexa 555-labeled secondary antibodies (dilution 1:100; Molecular Probes). F-actin was visualized with Alexa 594-phalloidin (dilution 1:100; Molecular Probes). As a negative control for transfection, cells were transfected with pFLAG-LacZ control vector. Immunofluorescence controls were run in transfected cells without the addition of primary antibodies. Immunofluorescence micrographs were obtained using a Nikon ECLIPSE E600 confocal microscope, coupled to a Laser Scan System Radiance 2100 camera (Biorad, Hercules, CA) driven by Laser Sharp 2000 software, using Nikon 60× PlanApo A/1.40 oil objective. To reduce bleeding through, double-label confocal images were acquired sequentially (xy and xz sections). To examine the degree of overlap of the red and green spots, analysis of pixel co-localization was performed, as described [Garbelli et al., 2008], using the co-localization function of Laser Sharp 2000 software. Analysis was carried out on the transfected cells of three different experiments. From each cellular image three distinct fields (regions of interest, ROIs) for each specific cellular compartment were sampled. Manders' overlap coefficient [Manders et al., 1993] was employed to evaluate co-localization. To assess the reliability of this method we applied the same procedure to Caki-1 cells double-stained for EphB2 and F-actin, that are two antigens that do not co-localize (negative control). For each cellular compartment an average (±SEM) of coefficients obtained from the examined fields was calculated. Student's *t*-test was used to assess whether co-localization indices (Manders' overlap coefficients) of Arg isoforms with F-actin or with microtubules

were significantly different from that of negative control groups. Statistical significance was set at $P < 0.05$.

RESULTS

QUANTITATIVE ANALYSIS OF ARG TRANSCRIPTS IN HEK293T AND CAKI-1 CELL LINES BY REAL-TIME PCR

In order to choose the adequate cellular model in which to investigate whether all the predicted full-length isoforms of Arg are effectively expressed, we quantified the total Arg transcripts and the different 5′- and 3′-ends in Caki-1 and Hek293T cell lines. The absolute quantification of total Arg transcripts was performed by Real-Time PCR and the amount of Arg transcript, expressed as mean ± SEM of molecules/g RNA, was in Hek293T cells $2.3 \times 10^3 \pm 1.2 \times 10^2$ and in Caki-1 cells $9.3 \times 10^3 \pm 0.4 \times 10^3$. This difference was significant ($P < 0.03$) and these Real-Time PCR data suggest a higher expression of total Arg transcripts in Caki-1 respect to Hek293T cell line.

The relative quantification of different 5′- and 3′-ends of Arg transcripts in Hek293T and Caki-1 cells by Real-Time PCR evidenced that the four 5′-ends of Arg transcripts were all expressed in the Caki-1 cell line although in different amounts (Fig. 2A). In fact, in Caki-1 cells the 1BL form of the 5′-ends was the most abundant, however, all the other forms (1AS, 1AL, and 1BS) were represented.

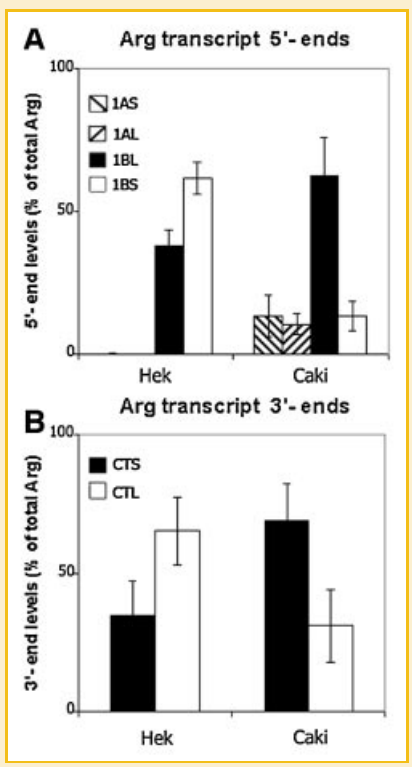

Fig. 2. Relative quantification of 5′-ends (A) and 3′-ends (B) of Arg transcripts in Hek293T and Caki-1 cell lines. The values, calculated as $2^{-\Delta CT}$, are expressed as percentage of the single end with respect to the total Arg transcripts. Mean ± SEM of three independent experiments performed in duplicate.

This expression pattern of 5′-end Arg transcripts of Caki-1 cells was different respect to the one previously published [Perego et al., 2005a], probably because, for this new set of experiments, we cultured a new batch of cryopreserved Caki-1 cells that showed slightly different karyotypic alterations with respect to those present in the cells previously used (data not shown). However, for our aim this discrepancy was not a contraindication, in fact, to prove the real existence and expression of the predicted eight full-length Arg transcripts the detectability of all 5′- and 3′-ends of Arg transcript and not their relative levels was important. In Hek293T cell line the 1A forms of the 5′-ends were unquantifiable and only the 1B forms were detected (Fig. 2A), with the 1BS 5′-end more abundant than 1BL.

The relative quantification of 3′-ends of Arg transcripts showed that both CTS and CTL forms were expressed in Hek293T and Caki-1 cells (Fig. 2B) with the CTL form predominant in Hek293T cells and CTS form in Caki-1 cells.

DETECTION OF EIGHT FULL-LENGTH ARG TRANSCRIPT ISOFORMS IN CAKI-1 CELL LINE BY REVERSE TRANSCRIPTION-LONG PCR

In the Caki-1 cells that express all 5′- and 3′-end transcripts we looked for the real presence of the predicted eight full-length Arg transcript isoforms as shown in Figure 1B. Total RNA extracted from the Caki-1 cell line was reverse transcribed by the specific reverse primer R (Fig. 1A) that recognizes a sequence just downstream the Arg stop codon. The obtained cDNA was amplified in four different Long-PCR reactions using four different primer sets (1/2; 1/3; 4/2; 4/3), as shown in Figure 1A. Electrophoresis on 1% agarose gel of the amplicons obtained showed the presence of full-length transcripts corresponding to the isoforms with exon 1A and different 3′-ends (1ACTL, 1ACTS) and to the isoforms with exon 1B and different 3′-ends (1BCTL, 1BCTS) (Fig. 3A, lanes a–d). This gel was not able to discriminate (into the amplicons) the bands corresponding to the full-length isoforms that respectively have or lack the exon II (long and short 5′-ends), which were only 63 bp different. So each product of the first round PCR was submitted to a second round of amplification using two different inner primer sets specific for the long and short 5′-ends of the 1A and 1B isoforms (Fig. 1A). The analysis on 1.8% agarose gel of the amplicons obtained (Fig. 3B, lanes a1–d2) evidenced the bands of expected size demonstrating that in the first round PCR all the 8 different 5′- and 3′-end combinations are present, and that all eight predicted full-length Arg transcripts are expressed in the Caki-1 cell line.

EVALUATION OF ENDOGENOUS ARG PROTEIN ISOFORMS BY 1-D AND 2-D WESTERN BLOTTING

The findings that eight Arg full-length transcript isoforms are present in the Caki-1 cell line supported the idea that also different Arg protein isoforms may be detected. 1-D Western blot of Hek293T and Caki-1 lysates assayed with polyclonal anti-Arg antibodies revealed a set of two bands in the range of 130–150 kDa (Fig. 4, lanes 4). Moreover, monoclonal anti-Arg antibodies detected in Hek293T and Caki-1 lysates only the slower but not the faster migrating protein band (Fig. 4, lanes 7). It is of note that these monoclonal antibodies have been raised against a peptide that contains part of the CT sequence, that is lost in the CTS isoforms. The difference

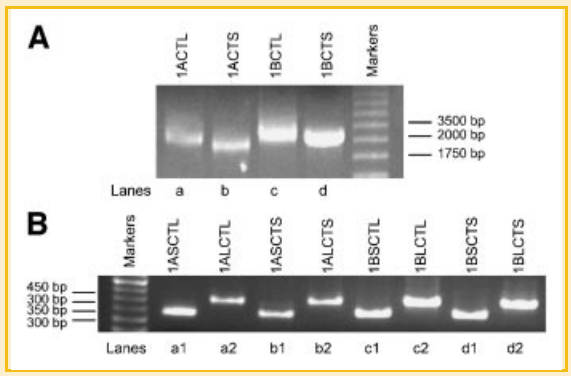

Fig. 3. Detection of the eight different full-length Arg transcript isoforms in Caki-1 cell line by Reverse Transcription-Long PCR. A: First round PCR products. The 1/2 primer pair (see Fig. 1A) amplified a large band corresponding to 1ACTL isoforms (lane a); the 1/3 primer pair revealed a large band corresponding to 1ACTS isoforms (lane b); the use of 4/2 primer pair led to obtain a large band corresponding to 1BCTL isoforms (lane c) and the use of 4/3 primer pair revealed a large band corresponding to 1BCTS isoforms (lane d). B: Second round PCR products. First round PCR products of lanes a and b amplified with primer pair 6/7 (see Fig. 1A) give rise to a 320 bp band corresponding respectively to 1ASCTL (lane a1) and 1ASCTS (lane b1) isoforms, and amplified with primer pair 5/7 give rise to a 400 bp band corresponding respectively to 1ALCTL (lane a2) and 1ALCTS (lane b2) isoforms. First round PCR products of lanes c and d amplified with primer pair 9/7 give rise to a 339 bp band corresponding respectively to 1BSCTL (lane c1) and 1BSCTS (lane d1) isoforms and amplified with primer pair 8/7 give rise to a 398 bp band, corresponding respectively to 1BLCTL (lane c2) and 1BLCTS (lane d2) isoforms.

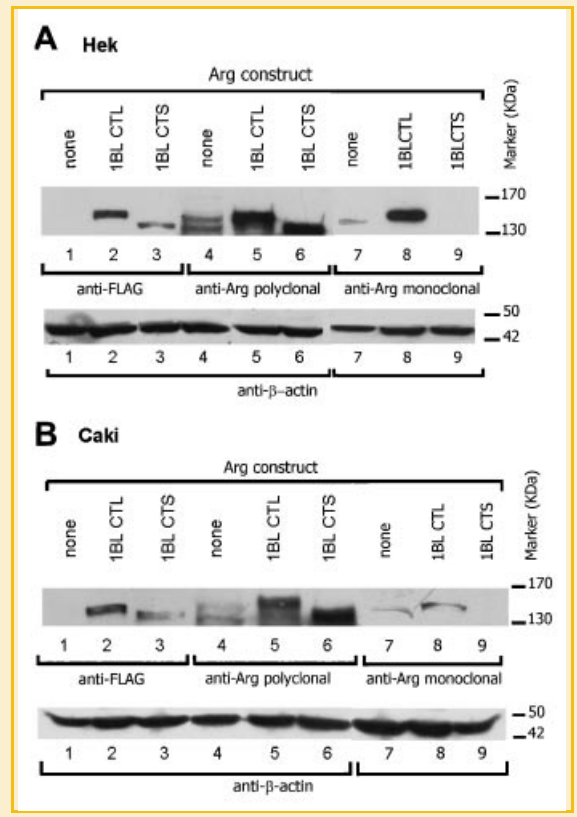

Fig. 4. 1-D immunoblot analysis of Hek293T and Caki-1 cell lysates untransfected or transfected by 1BLCTL and 1BLCTS Arg isoforms. Western blot analysis of 60 g of Hek293T (A) and Caki-1 (B) cell lysates, obtained from untransfected cells (lanes 1, 4, and 7) or 24 h after the transfection with pFLAG-CMV2 vector containing the indicated inserts (lanes 2, 3, 5, 6, 8, and 9). Anti-FLAG (lanes 1–3), polyclonal anti-Arg (lanes 4–6), monoclonal anti-Arg (lanes 7–9) and anti--actin (lanes 1–9) antibodies have been used.

corresponding to the N-termini, determined by exon IA or IB and by the presence or absence of exon II was not detectable on 1-D Western blot. Therefore, the ≃10 kDa different bands detected by polyclonal anti-Arg antibodies represent the full-length Arg protein isoforms with different C-termini. There is also quite a good agreement between protein band intensity and the relative amount of the 3′-end CTL and CTS transcripts evaluated by Real-Time PCR at least in Caki-1 cell lines. Indeed, the shorter protein was more abundant in Caki-1 lysate (Fig. 4B, lane 4), instead both proteins were roughly equivalent in Hek293T cells (Fig. 4A, lane 4). These data were confirmed by serial dilution of corresponding lysates analyzed by Western blot (data not shown). In the attempt to also detect the N-terminal different Arg protein isoforms, Hek293T and Caki-1 lysates were submitted to 2-D Western blot analysis using polyclonal anti-Arg antibodies. Different sets of spots, two in Hek293T and 4–5 in Caki-1, have been detected in the region in which high molecular weight and slight acidic (range 6–7 of isoelectric point) proteins migrate (Fig. 5).

ANALYSIS OF INTRACELLULAR DISTRIBUTION AND CYTOSKELETAL INTERACTIONS OF 1BLCTL AND 1BLCTS ARG ISOFORMS TRANSFECTED IN HEK293T AND CAKI-1 CELLS

To gain insight into the eventual different functions of Arg isoforms, we examined the subcellular localization of two after transfection. We subcloned the 1BLCTL and 1BLCTS Arg isoforms in pFLAG-CMV2 expression vector and transfected Hek293T and Caki-1 cells. The transfection efficiency obtained was 60% in Hek293T cells and

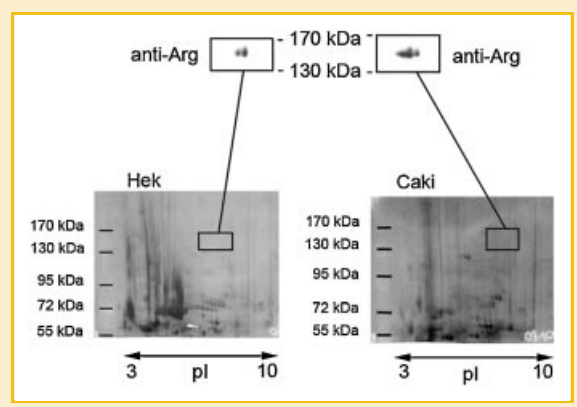

Fig. 5. 2-D immunoblot analysis of Hek293T and Caki-1 cell lysates. Amido Black stained 2-D blot membranes with the 2-D blot regions (highlighted by rectangles) in which polyclonal anti-Arg antibodies detected the different sets of spots shown in the corresponding square-zoomed areas of 2-D Western blots. Molecular weight markers and isoelectric point range are also indicated.

10% in Caki-1 cells for both constructs. The transiently transfected 1BLCTL and 1BLCTS isoforms were readily detected by anti-FLAG antibody in Hek293T and Caki-1 lysates (Fig. 4, lanes 2 and 3). Furthermore, in both cell lysates, polyclonal anti-Arg antibodies, directed against SH2-SH3 domains, detected the protein bands corresponding to both transfected isoforms and, in untransfected cell lysate, those corresponding to the endogenous protein (Fig. 4, lanes 4–6). In particular, in immunoblots probed with polyclonal anti-Arg antibodies, the protein band corresponding to transfected 1BLCTL and 1BLCTS isoforms migrate respectively as the slower and faster migrating endogenous protein band of Hek293T and Caki-1 cells (Fig. 4, lanes 5 and 6). Instead, monoclonal anti-Arg antibodies, directed against a C-terminal region including part of CT sequence, detected the protein band corresponding to 1BLCTL but not to the 1BLCTS transfected isoform (Fig. 4, lanes 8 and 9). Furthermore, the 1BLCTL transfected isoform migrates like the slower migrating band detected by these monoclonal antibodies in untransfected Hek293T and Caki-1 cell lysates (Fig. 4, lanes 7).

Cytoplasmic staining was detected by direct immunofluorescence using FITC-conjugated anti-FLAG antibodies in Caki-1 and Hek293T cells transfected with either 1BLCTL (Fig. 6A,C) or 1BLCTS (Fig. 6B,D) Arg isoforms. Both Arg isoforms (1BLCTL and 1BLCTS) co-localize with F-actin at the cell periphery in transfected Caki-1 (Manders' coefficient 0.978 ± 0.004 and 0.902 ± 0.011, respectively) and in Hek293T cells (Manders' coefficient 0.880 ± 0.041 and 0.723 ± 0.052, respectively). The degree of overlap of the signals corresponding to these antigens was significantly higher ($P < 0.002$) than the one obtained for two unrelated antigens (EphB2 and F-actin) in Caki-1 cells (Manders' coefficient 0.245 ± 0.036). It is of note that, in Hek293T cells only, the recombinant 1BLCTS isoform constantly gives rise to a cytoplasmic ring-like structure (Fig. 6D), in which there is no evidence of co-localization with F-actin (Manders' coefficient 0.394 ± 0.047; $P = 0.083$). However, phalloidin-positive amorphous actin aggregates are inside the ring (Fig. 6D).

To better define the molecular composition of this ring-like structure, the microtubule distribution has been also analyzed. In Hek293T cells transfected with the 1BLCTL isoform, Arg co-localizes with microtubules throughout the cytoplasm (Manders' coefficient 0.909 ± 0.007; $P < 0.001$) and at the cell periphery (Manders' coefficient 0.911 ± 0.010; $P < 0.001$) (Fig. 7A). When Hek293T cells are transfected with 1BLCTS isoform the diffuse cytoplasmic co-localization of Arg with microtubules was no longer detectable. It remained detectable only at the periphery of the cell (Manders' coefficient 0.857 ± 0.016; $P < 0.001$). In this latter case 1BLCTS and microtubules do not co-localize at the Arg-rich ring-like structure (Manders' coefficient 0.359 ± 0.053; $P = 0.164$) and -tubulin-positive aggregates are not detectable inside the ring (Fig. 7B). No specific cellular distribution or co-localization with the cytoskeletal proteins were detectable in Caki-1 and Hek293T cells transfected with pFLAG-LacZ control vector.

The topographical characterization of the ring-like structure detected in Hek293T cells transfected with 1BLCTS isoform has been improved by confocal scanning microscopy analysis in the horizontal sections (x,y) (Fig. 8A,B) and corresponding vertical sections (x,z) (Fig. 8A′,B′) of the immunodetected transfected cells. It is clear that the 1BLCTS Arg isoform gives rise to a hollow structure crossing the cytoplasm from the apical to the basal surface (Fig. 8A′,B′). F-actin aggregates may be detected in the cavity of this structure (Fig. 8A,A′) whereas microtubules are not (Fig. 8B,B′).

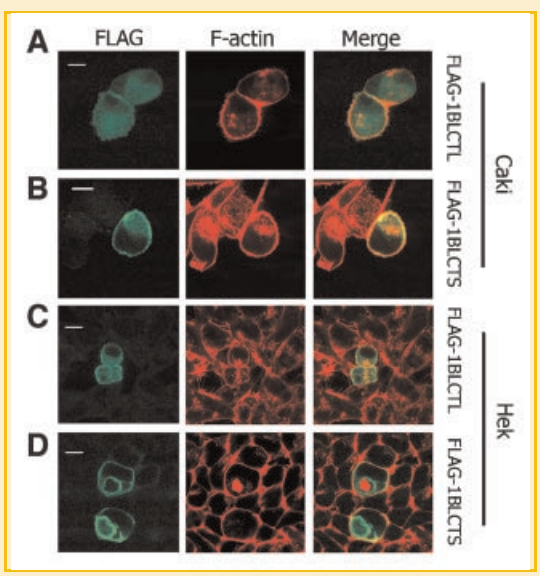

Fig. 6. Cellular distribution and co-localization with F-actin of transfected Arg isoforms. Caki-1 cells over-expressing FLAG-1BLCTL (A) and FLAG-1BLCTS (B) and Hek293T cells over-expressing FLAG-1BLCTL (C) and FLAG-1BLCTS (D). Merge images (yellow) show the co-localization of FLAG-Arg (green) with F-actin (red) at the cell periphery. Bar, 10 m. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

DISCUSSION

Two isoforms of both human Arg and c-Abl have been described as having different N-termini called 1A and 1B [Shtivelman et al., 1986; Kruh et al., 1990]. Four mouse c-Abl transcript isoforms with different 5′-ends arising as a result of addition of alternative 5′-exons have been cloned [Bernards et al., 1988]. More recently, we have shown the presence of four different 5′- and two 3′-ends of Arg transcripts in human cells. Combining these different 5′- and 3′-ends

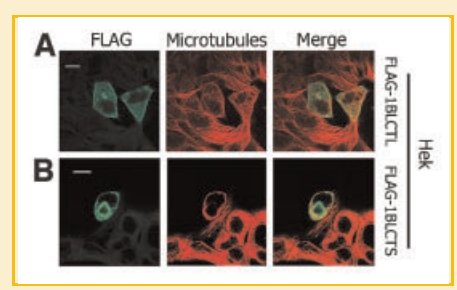

Fig. 7. Co-localization with microtubules of transfected Arg isoforms. Hek293T cells transfected with FLAG-1BLCTL (A) and FLAG-1BLCTS (B). Merged images (yellow) show the co-localization regions of FLAG-Arg (green) with microtubules (red). Bar, 10 m. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

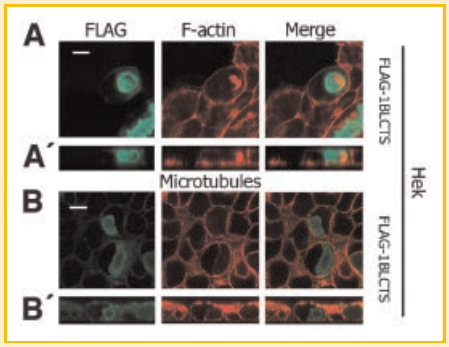

Fig. 8. Topographical distribution of FLAG-1BLCTS Arg transfected in Hek293T cells and co-localization with F-actin and microtubules. FLAG-Arg (green) and F-actin (red) immunolocalization in horizontal x,y (A) and vertical x,z (A') cellular sections. FLAG-Arg (green) and microtubules (red) immunolocalization in horizontal x,y (B) and vertical x,z (B') cellular sections. Merge images in yellow. Bar, 10 m. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

it was possible to predict eight full-length Arg transcripts and eight putative proteins [Perego et al., 2005a]. To investigate whether all predicted eight full-length transcripts were really expressed in human cells, we analyzed the RNA extracted from the Caki-1 cell line that had all different 5′- and 3′-end Arg transcripts. Moreover, absolute quantification of total Arg transcripts by Real-Time PCR showed that Arg transcript expression was up to 4 times higher in renal cancer Caki-1 than in embryonic kidney Hek293T cell line in which not all Arg transcript ends were expressed. The presence of all different 5′- and 3′-ends of Arg transcripts and the relative high expression of total Arg transcripts in Caki-1 cells compared to other cell lines previously analyzed [Perego et al., 2005a] were the major reasons why we used this cell line to look for the endogenous presence of all full-length Arg isoforms. Hek293T cells were chosen instead for their easy transfectability.

We demonstrated here for the first time that all eight putative full-length Arg transcripts are endogenously expressed, at least in the Caki-1 cell line, by Reverse Transcription-Long and nested PCR. The predicted corresponding Arg protein isoforms, all expected in Caki-1 cells based on transcript data, have also been investigated. We demonstrated with 1-D Western blot and polyclonal anti-Arg antibodies that the proteins corresponding to 1BLCTL and 1BLCTS transfected Arg isoforms co-migrate with the slower and faster endogenous Arg protein bands, respectively. Furthermore, the monoclonal anti-Arg antibodies, that we showed are able to recognize only the full-length CTL isoforms, detected only the slower protein band in untransfected cell lysates. All these data provide compelling evidence that the ≃10 kDa different bands detected by Western blot in both Hek293T and Caki-1 cells correspond to the full length Arg protein isoforms with different C-termini. The size difference determined by the alternative presence or absence of 21 amino acids, coded by exon II, was not sufficient to reveal the isoforms that differ only at N-termini by monodimensional electrophoresis. Therefore, we tried to identify the different Arg isoforms by 2-D Western blot. The different sets of high molecular weight and slight acidic spots detected by polyclonal anti-Arg antibodies in Hek293T and Caki-1 lysates may correspond to some Arg isoforms. These spots may represent the isoforms expressed at the highest level in the two different cell lines. Alternatively, they may also correspond to all different full-length Arg isoforms expressed in these cell lines, although they may well not be separated along the first and/or second dimension of 2-D electrophoresis. Our attempts to further identify the different Arg isoforms by 2-D electrophoresis and mass spectrometry analysis were unsuccessful because of the low abundance of endogenous Arg proteins, whose protein spots were detectable only by 2-D Western blot. Anyway, based on our 1-D Western blot data, it is possible to argue that full-length isoforms with CTS form in the C-termini prevail in Caki-1 cells, and the full-length isoforms may have four possible N-termini (1AS, 1AL, 1BS, 1BL), as shown by our Real-time PCR data. In Hek cells the full-length isoforms with CTS and CTL forms in the C-termini are roughly equally expressed, but in these cells the full-length isoforms may have only the 1BL and 1BS N-termini.

Our aim was not to define the role of Arg isoforms in normal and neoplastic renal cells, however, our previous data [Perego et al., 2005a] showed that the expression pattern of 5′- and 3′-ends of Arg transcripts is correlated to normal and malignant cell type and may also change during cell differentiation. Therefore, the embryonic nature of Hek293T cells and the neoplastic characteristics of Caki-1 cells might be responsible of the different expression pattern of 5′- and 3′-transcript ends in these two renal cell lines, suggesting for Arg isoforms with different N- and C-termini a possible different role in renal cells, although it is still obscure. In fact, the function of the first exons A and B is not yet well defined, although exon B can be miristoylated [Kruh et al., 1990] and this favors its membrane anchorage. Furthermore, the presence of exon II in Arg protein seems to be important for protein activation as shown in the chimeric oncogenic protein Tel/Arg [Iijima et al., 2002]. Interestingly, recent data have shown [Peacock et al., 2007] that Arg requires distinct functional domains to inhibit fibroblastic focal adhesions and actomyosin contractility. The N-terminal half of Arg containing the kinase domain can act to attenuate stress fiber formation and cell contractility. However, Arg requires both its kinase activity and its cytoskeleton-binding C-terminal half to fully inhibit focal adhesions.

In this article, we also described the subcellular localization of 1BLCTL and 1BLCTS Arg isoforms transfected in Caki-1 and Hek293T cells. The 1BLCTL isoform, that is the only Arg isoform currently used in literature for functional studies, has been compared with 1BLCTS isoform, that differs only in the C-terminal domain, checking whether the partial loss of the internal F-actin binding domain of this latter isoform changes its cellular localization or cytoskeleton interactions. Cytoplasmic staining was detected in both cell types transfected with 1BLCTL or 1BLCTS isoforms that co-localized with cortical F-actin. Of note, in literature an Arg1B deletion mutant, completely lacking the internal F-actin binding domain, failed to co-localize with F-actin at the cell periphery when transfected in Swiss 3T3 cells, differently from wild-type Arg1B (the 1BLCTL isoform) [Wang et al., 2001]. Moreover, in Hek293T cells, only the transfected 1BLCTS isoform gives rise to a hollow structure that crosses the cytoplasm from the apical to the

basal surface and does not co-localize with F-actin that, instead, aggregates inside the hollow portion. This peculiar topographic distribution of the 1BLCTS Arg isoform in Hek293T cells could be related to some cytological or molecular characteristics specific of this cell type like, for example, the lack of distinct stress fibers [Lawrenson et al., 2002; Asanuma et al., 2005]. This peculiar organization of Hek293T cell cytoskeleton might perhaps influence this distribution pattern of the 1BLCTS isoform, given the documented role of Arg in the modulation of stress fibers [Peacock et al., 2007]. Thus, the partial deletion of the internal F-actin binding domain in the 1BLCTS isoform is not enough to alter the F-actin bundling property but might influence the cytosolic distribution of Arg protein in absence of stress fibers. Moreover, the 1BLCTL Arg isoform co-localizes with microtubules throughout the cytoplasm and at the cell periphery in transfected Hek293T cells, as described in transfected $Arg^{-/-}$ fibroblast [Miller et al., 2004]. The lack of CT sequence in the 1BLCTS Arg isoform modifies the cytoplasmic co-localization pattern with microtubules, maintaining the capacity to co-localize at the cell periphery. Also in this case, no co-localization signal was detectable at the Arg-rich hollow structure. The cellular expression and localization data described suggest that likely the diverse full-length Arg isoforms might have different functional implications in Caki-1 and Hek293T cells. In fact, their different expression patterns may give rise to different concentrations of kinase activity, differently modulated by the different N-terminal domains, in the subcellular compartments through the different cytoskeleton interactions mediated by the different C-termini. All this may results in different physiological or pathological effects and our demonstration that eight full-length Arg isoforms are effectively present in the cells with different concentrations will make possible future studies referred to the role of the single full-length isoforms even in malignant renal cells. These cells could be conveniently studied in primary cultures, of normal and tumor human renal tissue, that are routinely established and well characterized in our laboratory [Perego et al., 2005b] and that simulate the in vivo situation most closely than corresponding cell lines.

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