FEBS Letters 539 (2003) 78–84 FEBS 27076

Interaction between HERC1 and M2-type pyruvate kinase

Francesc R. Garcia-Gonzalo[a], Cristina Cruz[a], Purificación Muñoz[a], Sybille Mazurek[b], Erich Eigenbrodt[b], Francesc Ventura[a], Ramon Bartrons[a], Jose Luis Rosa[a,*]

[a] *Departament de Ciències Fisiològiques II, Campus de Bellvitge, Universitat de Barcelona, C/Feixa Llarga s/n, E-08907, L'Hospitalet de Llobregat, Barcelona, Spain*

[b] *Institut für Biochemie und Endokrinologie, Fachbereich Veterinärmedizin, Justus Liebig Universität, Frankfurter Strasse 100, D-35392 Giessen, Germany*

Received 18 December 2002; revised 16 February 2003; accepted 18 February 2003

First published online 5 March 2003

Edited by Ulrike Kutay

Abstract HERC proteins are characterized by having one or more RCC1-like domains as well as a C-terminal HECT domain in their amino acid sequences. This has led researchers to suggest that they may act as both guanine nucleotide exchange factors and E3 ubiquitin ligases. Here we describe a physical interaction between the HECT domain of HERC1, a giant protein involved in intracellular membrane traffic, and the M2 isoform of glycolytic enzyme pyruvate kinase (M2-PK). Partial colocalization of endogenous proteins was observed by immunofluorescence studies. This interaction neither induced M2-PK ubiquitination nor affected its enzymatic activity. The putative significance of the association is discussed.

Key words: HERC1; M2-type pyruvate kinase; HECT domain; RCC1-like domain; E3 ubiquitin ligase; Guanine nucleotide exchange factor

1. Introduction

The human HERC protein family consists of four proteins sharing a HECT domain in their carboxyl-termini and one or more RCC1-like domains (RLDs) elsewhere in their amino acid sequences. The HECT domain (homologous to E6-AP carboxyl-terminus) was first characterized as the domain involved in the E6-AP-mediated ubiquitination of p53 in the presence of papillomaviral E6 oncoprotein [1]. More generally, HECT domains are assumed to confer E3 ubiquitin-protein ligase activity to those proteins containing them through a mechanism involving formation of a thioester bond between ubiquitin's carboxyl-terminus and a conserved active site cysteine residue in the HECT [2,3]. The RCC1-like domain, on the other hand, was initially identified in the regulator of chromosome condensation-1 (RCC1) protein, where it has been shown to stimulate GDP/GTP exchange upon Ran, a monomeric G-protein belonging to the Ras superfamily of GTPases, and thus to regulate important cellular processes such as nucleocytoplasmic transport and mitotic spindle formation [4,5]. For this reason, it is thought that RLDs may act as guanine nucleotide exchange factors (GEFs) for small GTPases [6].

The HERC family can in turn be divided into two subfamilies: the large HERCs (HERC1 and HERC2) are giant proteins almost 5000 amino acid residues long, encoded in chromosome 15 [7] and possessing at least two RLDs and other known sequence motifs in addition to the HECT, whereas the small HERCs (HERC3 and HERC4) are less than one fourth the size of their larger counterparts, are encoded in chromosome 4 [8] and their primary structures display little more than one RLD and the HECT. Very little is known about the cellular functions of these proteins. Mouse HERC2, encoded in the *rjs/jdf2/herc2* locus, has been linked to a genetic syndrome whose major symptoms include dwarfism, a jerky gait and sterility. Interestingly, all these phenotypes appear to be due to the loss of function of HERC2's HECT domain [9,10]. HERC3 is a cytosolic and inner membrane-associated protein which has recently been shown to bind non-covalently to ubiquitin as well as to undergo ubiquitin-mediated proteasomal degradation [11]. HERC4 (also called Ceb-1) was isolated in a yeast two-hybrid screening using both cyclin E and p21 as baits. Unlike all other HERCs whose expression is rather ubiquitous, HERC4 is selectively expressed in reproductive tissues and undergoes upregulation when the functions of both p53 and pRB tumor suppressors are compromised [12]. HERC1, the largest family member (532 kDa) and the first to be described, contains a number of conserved sequence features which are supposed to play different roles in the overall function(s) of the protein. HERC1 possesses two RLDs (RLD1 and RLD2) which have been implicated in different cellular tasks: while RLD1 stimulates guanine nucleotide dissociation on several ARF and Rab family GTPases [6], RLD2 forms a ternary complex with clathrin heavy chain and the chaperone Hsp70 [13]. These data, together with HERC1's localization in inner cell membranes such as the Golgi apparatus and the cytosol [6], have led to the suggestion that this protein has an important function in intracellular membrane traffic. In addition to its RLDs, the most relevant conserved regions in HERC1 include a SPRY (repeats in splA and RyR) domain, a WD-40 domain, both thought to mediate protein–protein interactions [14,15], an F-box motif that might account for HERC1 being a constituent of a so far unidentified

*Corresponding author. Fax: (34)-934024268.
E-mail address: rosa@bellvitge.bvg.ub.es (J.L. Rosa).

Abbreviations: aa, amino acid residues; PBS, phosphate-buffered saline; GST, glutathione-S-transferase

0014-5793/03/$22.00
doi:10.1016/S0014-5793(03)00205-9

SCF E3 ubiquitin ligase complex [15,16], three putative SH3 binding sites and, finally, the HECT domain, which has already been shown to bind ubiquitin in a dithiothreitol-sensitive manner, thus confirming its ability to act as an E3 ubiquitin-protein ligase [17].

All the above-stated conserved regions present in HERC1 have already been involved in the formation of heteromolecular complexes through protein–protein interactions. In order to identify proteins that interact with HERC1, we have used several HERC1 domains as baits in the yeast two-hybrid system. In this study, we report the identification of M2-type pyruvate kinase as a protein that interacts with the HECT domain of HERC1.

2. Materials and methods

2.1. Plasmids

The 5′-untranslated region of HERC1 cDNA was removed from previously described plasmid pJLR75 [6] by first amplifying a polymerase chain reaction (PCR) fragment in the 5′-coding region with oligos SEQJL135 (5′-CCATCGATGAATTCAACATGGCAACTATGATTCCA-3′) and SEQJL057 (5′-CTTCTCCGAAAGAGGCCATA-3′) and then ligating the *Cla*I–*Nde*I-digested PCR fragment with the larger fragment resulting from digestion of pJLR75 with the same restriction enzymes. This new plasmid was called pFG1 and was used to generate pFG3 by subcloning its *Eco*RI insert into pcDNA3.1/HisC (Invitrogen). pJLR82 was created by cloning into pGBT9 (Clontech) a *Bam*HI–*Sal*I-digested DNA fragment obtained by PCR amplification of pJLR75 with oligos SEQJL118 (5′-CGGGATCCCGAGACAAGTAGTTAAGCTG-3′) and SEQJL119 (5′-ACGCGTCGACGGTCAGTAGTCAGTGTCG-3′). *Eco*RI–*Sal*I insert was extracted from pJLR82 and subcloned into both *Eco*RI–*Sal*I-digested pFastBacHTa (Invitrogen) and *Eco*RI–*Xho*I-cut pcDNA3.1/HisC to form plasmids pFG26 and pCC44, respectively. pFastBacHTa digested with *Rsr*II and *Eco*RI was ligated with a DNA fragment produced by PCR-amplifying vector pGEX4T1 (Amersham Pharmacia Biotech) with oligos RsrIIGST (5′-CTCGGTCCGAAACCATGTCCCCTATACTAGGT-3′) and GSTThr (5′-GGGAATTCCGGGGATCCACGCGGAACCAG-3′) followed by digestion with the same restriction endonucleases. The resulting plasmid, pFastBacGSTa, was subsequently used to create another plasmid, pPM7, by introducing into the former the *Eco*RI insert from pFG1. pJDD7 was similarly created through ligation of the 4 kb *Bam*HI–*Eco*RI fragment of pFG1 with *Bam*HI–*Eco*RI-restricted pFastBacGSTa. pJDD8 and pJDD9 were generated by digesting pPM7 with either *Spe*I (pJDD8) or *Xho*I (pJDD9) followed by religation of the vector-containing fragments. pFG32 was obtained by transferring the *Sma*I–*Xho*I insert from pClone25 (see Section 3) into pGEX4T1. pT7-7-His-UbcH5a was provided by Dr. Kazuhiro Iwai.

2.2. Yeast two-hybrid experiments

Yeast experiments were carried out according to Matchmaker Gal4 Yeast Two-Hybrid System-3 (Clontech). Briefly, *Saccharomyces cerevisiae* AH-109 cells were cotransformed with pJLR82 (encoding HERC1's last 366 amino acid residues) and a HeLa cell cDNA library cloned into pGAD-GH vector (Clontech). Transformants were seeded on appropriate selective media in order to isolate positive clones and library plasmids were isolated from these by complementation of the leuB6 mutation of *Escherichia coli* MH4 strain. For filter β-galactosidase assays see [13].

2.3. Antibodies

Mouse monoclonal anti-M2-PK (clone DF4) was from ScheBo Biotech (Giessen, Germany). Mouse monoclonal anti-His (clone His-1) and anti-glutathione-S-transferase (GST) were from Sigma, as was rabbit anti-ubiquitin antiserum. Affinity purified rabbit polyclonal antibodies against HERC1 (410 and 417) have already been described elsewhere [6].

2.4. Baculoviruses, protein purification and pull-down experiments

2.4.1. Baculoviruses. Recombinant baculoviruses expressing His-HECT, GST, GST-HERC1 (amino acid residues (aa) 1–1413) GST-HERC1 (aa 1–3716), GST-HERC1 (aa 1–4861, full length) and GST-HERC1 (aa 3684–4861) were generated from plasmids pFG26, pFastBacGSTa, pJDD9, pJDD8, pPM7 and pJDD7, respectively. All these baculoviruses were produced according to the Bac-to-Bac system's instructions manual (Invitrogen). M2-PK baculovirus was provided by Dr. Tamio Noguchi.

2.4.2. Protein purification. Nickel-NTA agarose beads (Qiagen) were used to purify both His-UbcH5a from *E. coli* BL21 cells transformed with plasmid pT7-7-His-UbcH5a and His-HECT from baculovirus-infected Sf9 cells. Likewise, GST-M2-PK (aa 406–531) expressed by pFG32-transformed *E. coli* XL1 blue cells, GST and GST-HERC1 (full length) from Sf9 cells were isolated using glutathione-Sepharose 4B beads (Amersham Pharmacia Biotech). Bacterial purifications were carried out according to standard procedures. Insect cell purifications were done basically as described in [6].

2.4.3. Pull-down experiments. In vitro pull-down experiments were performed by mixing in phosphate-buffered saline (PBS) 0.5 μg of either GST or GST-M2-PK (aa 406–531), both bound to glutathione-Sepharose beads, and 1.2 μg of soluble His-HECT. After 3 h of rocking at 4°C, beads were washed three times in PBS and then subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE)/immunoblot analysis. In vivo pull-down experiments were carried out 72 h after Sf9 cells had been infected with appropriate baculoviruses. Cells were lysed in ~1 ml/5×10^6 cells of buffer containing 20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1% (v/v) Nonidet P-40, 5 mM β-mercaptoethanol, 5 mM imidazole, 1 mM phenylmethylsulphonyl fluoride, 1 μg/ml pepstatin A, 5 μg/ml aprotinin and 5 μg/ml leupeptin and centrifuged 20 min at 15 000×*g*. 50 μl of either glutathione-Sepharose or nickel-NTA-agarose beads per ml supernatant were then added and the resulting mixture was rocked for 2 h. Beads were washed three times with lysis buffer containing 300 mM NaCl and once with lysis buffer (no protease inhibitors in washes). Pulled-down proteins were studied by SDS–PAGE/immunoblot. The pull-down in Fig. 1E was performed by mixing 100 ng of bead-bound, purified GST or GST-HERC1 and 5 ml of an M2-PK-expressing Sf9 cell lysate (lysis as above). The mixture was rocked for 2 h and beads washed four times with lysis buffer before being analyzed by SDS–PAGE/immunoblot.

2.5. Cell culture and transfection

HEK-293T and HeLa cells were maintained as described in [11]. Transfection of HEK-293T was achieved using the calcium phosphate method when cells were at a confluence of 40–70%. Experiments were performed 40 h after cells were transfected. Sf9 cells were cultured in Petri dishes at 27°C with insect cell medium (BioWhittaker) to which 10% fetal bovine serum was added. Baculovirus infections were carried out using standard procedures.

2.6. Confocal immunofluorescence microscopy

HeLa and HEK-293T cells were grown on glass coverslips and processed as described in [11]. Dilutions used were 1/25 and 1/200 for anti-M2-PK and anti-HERC1 (410) antibodies, respectively. Secondary antibodies were fluorescein-isothiocyanate (FITC)-conjugated goat anti-rabbit IgGs and Texas red-isothiocyanate-conjugated goat anti-mouse IgGs (both from Amersham Pharmacia Biotech). Samples were observed under a Leica TCS-NT confocal microscope.

2.7. Ubiquitination experiments and enzyme activity measurements

LLnL experiments in HEK-293T cells were performed as previously described [11]. In order to measure pyruvate kinase activity in HEK-293T cells, these were first placed on ice, washed once with ice-cold PBS and extracted in a buffer containing 10 mM Tris–HCl, pH 7.4, 1 mM NaF, 1 mM ethylenediamine tetraacetic acid (EDTA) and 1 mM β-mercaptoethanol. Cells were then lysed with a cell homogenizer and total cell extracts centrifuged 20 min at 15 000×*g*. Supernatants were then taken and used both to measure total protein levels (BCA kit, Pierce) and to carry out activity assays. These were done in a buffer containing 50 mM Tris–HCl, pH 7.6, 100 mM KCl, 5 mM $MgSO_4$, 2 mM ADP, 0.2 or 2 mM phosphoenolpyruvate (PEP), 0.25 mM NADH and 0.15 mg/ml lactate dehydrogenase (Roche Molecular Biochemicals). Activity was calculated by monitoring the absorbance fall at 340 nm. The effect of UbcH5 on M2-PK activity (Fig. 3C) was determined by adding 2 ng of His-UbcH5a per μg total protein to the HEK-293T extracts followed by a 30-min incubation at 4°C prior to activity measurements.

3. Results

3.1. HERC1–M2-PK interaction in the yeast two-hybrid system

A yeast two-hybrid screening was performed using the last 366 amino acid residues of HERC1 (aa 4496–4861), encompassing its HECT domain, as bait. Plasmid pJLR82 encoding the aforementioned HERC1 fragment fused to Gal4 DNA binding domain was transformed into *S. cerevisiae* AH-109 cells together with a library constructed by inserting HeLa cell cDNAs into *Eco*RI–*Xho*I targets of vector pGAD-GH, which encodes Gal4 transcriptional activation domain. Positive clones were selected by their ability to grow in the absence of histidine, adenine, leucine and tryptophan. Most positives obtained in this manner turned out to possess a library insert corresponding to the C-terminal region (aa 406–531) of glycolytic isoenzyme pyruvate kinase type M2. This interaction was confirmed by a filter β-galactosidase assay (Fig. 1A). The fact that M2-PK failed to interact with HERC3, a homologue of HERC1, appears to indicate that this interaction is specific for HERC1 and does not take place with other HERC family members. On the other hand, neither pJLR82 nor pClone25 (pGAD-GH with the M2-PK insert) gave rise to β-gal$^+$ cells when transformed together with empty pGAD-GH or pGBT9 vectors, respectively.

3.2. In vitro pull-down experiments

In order to confirm the yeast two-hybrid interaction as well as to check whether this was direct or else might be mediated by a bridging protein, histidine-tagged HERC1 (aa 4496–4861, henceforth also referred to as His-HECT) and GST-M2-PK (aa 406–531) fusion protein were purified and pull-downs were carried out with glutathione-Sepharose beads (Fig. 1B, see also Section 2). Results show clearly that His-HECT binds to GST-M2-PK (406–531) and does not to GST, from which it can be concluded that the interaction exists and is likely to be direct between the two proteins.

3.3. In vivo pull-down experiments in insect cells

Next, HERC1–M2-PK interaction was studied in vivo in baculovirus-infected Sf9 insect cells. First, cells were infected with baculoviruses encoding full-length M2-PK and His-HECT and lysates pulled-down with nickel beads (Fig. 1C). When this was done, a fraction of M2-PK was found in the beads, whereas none could be found when pull-downs were carried out in control cells expressing only M2-PK and not His-HECT, indicating that M2-PK bound specifically to His-HECT. Second, full-length M2-PK was heterologously expressed either alone or together with GST or three GST fusion proteins encompassing the entire length of HERC1 and cell extracts were pulled-down with glutathione beads. As expected, M2-PK could only be detected in the beads when it was coexpressed with the fusion protein containing HERC1's carboxyl-terminal region (GST-HERC1 (3684–4861)) and not in all other conditions (Fig. 1D). Finally, we attempted to find the interaction between both full-length proteins. In order to achieve this goal, we incubated purified, bead-bound GST or GST-HERC1 (aa 1–4861, full-length protein) with an M2-PK baculovirus-infected Sf9 cell lysate. Immunoblot analysis showed that a small fraction of M2-PK bound to GST-HERC1 beads, whereas none could be found in control GST beads (Fig. 1E). Taken together, these results demonstrate that M2-PK and HERC1, through its HECT domain, have the ability to interact with each other.

3.4. Immunofluorescence analysis of HERC1–M2-PK colocalization

Subcellular localization of endogenous HERC1 and M2-PK proteins was studied by indirect immunofluorescence confocal microscopy in HeLa and HEK-293T cell lines (Fig. 2). It is noteworthy that both proteins display a similar, mostly perinuclear, punctate staining, which probably means that these proteins somehow interact with membranous intracellular structures in these cell lines [18]. Furthermore, colocalization analysis shows a partial overlapping in the subcellular distributions of both proteins, as seen by the appearance of yellow dots when stainings are superimposed.

3.5. Analysis of M2-PK ubiquitination

In view of the fact that HERC1 is very likely to be an E3 ubiquitin-protein ligase (it possesses a HECT domain and an F-box motif, both generally recognized as hallmarks of such enzymes), we checked whether M2-PK might be a ubiquitination substrate of HERC1. Nevertheless, since ubiquitination of M2-PK has not been described, we first set about determining whether M2-PK undergoes ubiquitination under physiological conditions. For this purpose, we analyzed the effect of proteasome inhibitor *N*-acetyl-leucyl-leucyl-norleucinal (LLnL) on M2-PK protein levels in HEK-293T cells (Fig. 3A). While LLnL induced the accumulation of ubiquitinated forms of other proteins, this was not observed for M2-PK nor were its levels affected, as would have been expected of a proteasomally degraded protein. These results notwithstanding, the possibility remained that M2-PK underwent non-proteasome-coupled ubiquitination. To test this hypothesis, histidine-tagged ubiquitin was transfected into HEK-293T cells and cell lysates were incubated with nickel beads to pull-down His-ubiquitinated proteins. Immunoblot analysis showed that M2-PK was not among them (data not shown). Moreover, transcription and translation of radiolabeled M2-PK in rabbit reticulocyte lysates followed by addition of GST-ubiquitin did not result in formation of GST-ubiquitin–M2-PK adducts (data not shown). All results, therefore, point to the direction that M2-PK does not undergo ubiquitination and thus cannot be a ubiquitination substrate of HERC1.

3.6. Effect of HERC1 upon M2-PK enzyme activity

In order to analyze whether the HECT domain of HERC1 might modulate M2-PK enzyme activity, HEK-293T cells were transfected with either control plasmid (pcDNA3.1/His) or plasmid encoding His-HECT (pCC44). His-HECT expression was confirmed by immunoblot (data not shown). Activity was measured in cell lysates at two different phosphoenolpyruvate (PEP) concentrations: 0.2 mM (corresponding roughly to the concentration at which M2-PK has half-maximal activity ($S_{0.5}^{PEP} = 0.25$ mM) [19]) and 2 mM, a saturating PEP concentration. In this manner, we intended to distinguish between effects upon M2-PK's affinity for PEP and effects upon its V_{max}. However, as it can easily be grasped from Fig. 3B, none of those effects were observed. It might be, though, that the HECT domain alone is not sufficient to induce any alterations in M2-PK activity and that full-length HERC1 is needed for them to occur. To test this, we measured M2-PK activity in extracts from control and HERC1-

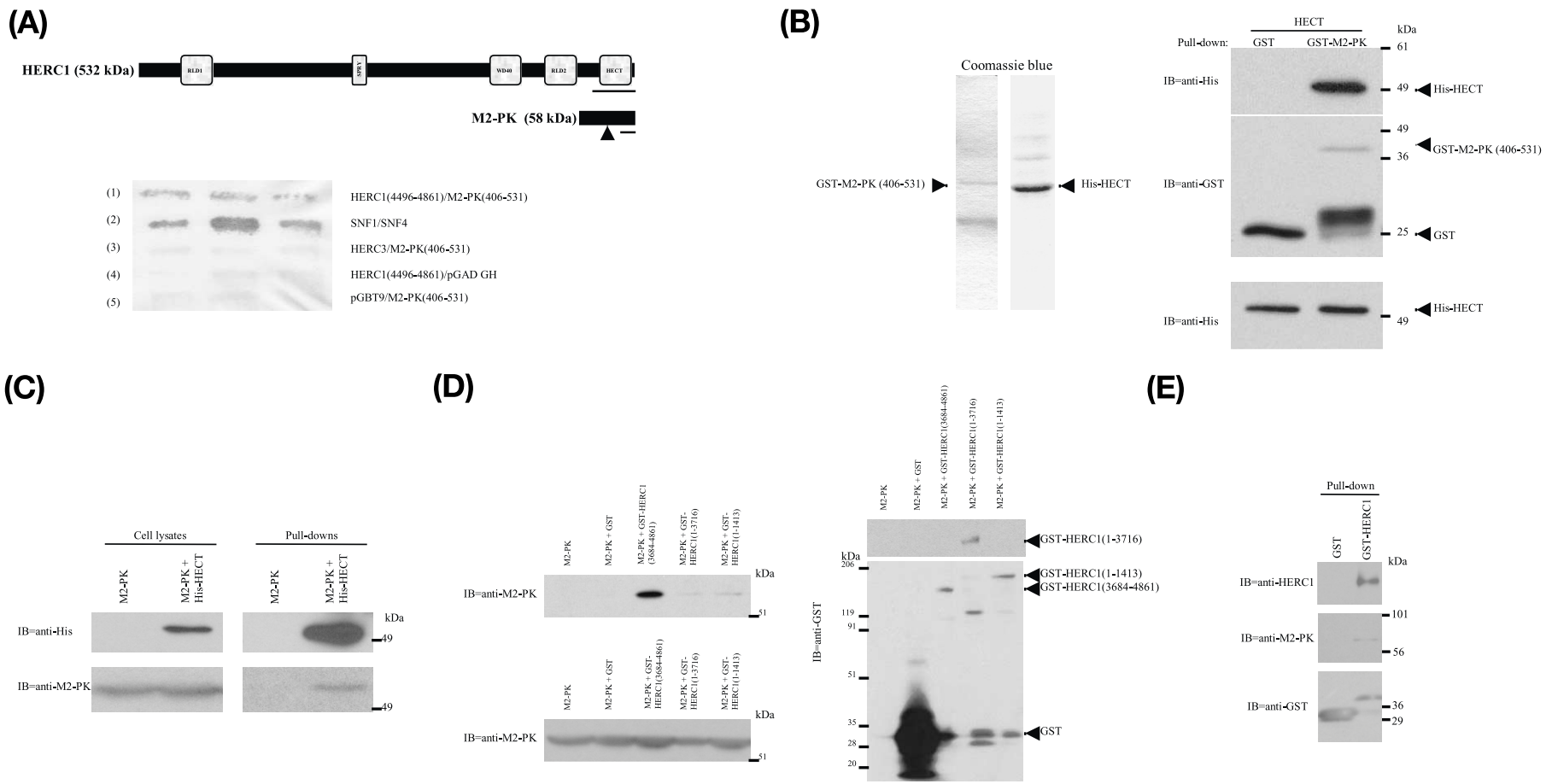

Fig. 1. Interaction between HERC1 and M2-PK. A: The last 366 amino acid residues of HERC1 were used as bait in the yeast two-hybrid system. Among all positive clones analyzed, most corresponded to the last 126 amino acid residues of pyruvate kinase type M2. Top: Schematic representations of HERC1 and M2-PK proteins have been drawn to scale. Major HERC1 domains have also been depicted. M2-PK's active site is marked with an arrowhead. Interacting regions in both proteins have been underlined. Bottom: Filter β-galactosidase assay. Three independent clones are shown for each condition. (1) Interaction between HERC1 (aa 4496–4861) and M2-PK (aa 406–531). (2) SNF1–SNF4 interaction as positive control. (3) Interaction is specific for HERC1, since it doesn't take place with HERC3. (4–5) Negative controls. B: Left: Coomassie blue staining showing purifications of GST-M2-PK (406–531) and His-HECT proteins. The lower band in the former is a degradation product. Right: In vitro pull-downs confirmed the yeast interaction. Pulled-down GST and GST-M2-PK (406–531) and the His-HECT associated to them were resolved by SDS–PAGE and analyzed by immunoblot. Bottom right: 4% of the His-HECT used in pull-downs was loaded into another gel and immunoblotted as well. C: Immunoblots of Sf9 cells infected with baculoviruses encoding full-length M2-PK and His-HECT as indicated. When His-HECT was pulled-down with nickel beads, M2-PK was also found associated. D: Recombinant baculoviruses expressing M2-PK, GST and three GST fusion proteins encompassing the whole HERC1 protein were used to infect Sf9 cells as indicated. Cell extracts were pulled-down with glutathione beads and associated proteins analyzed by SDS–PAGE/immunoblot. M2-PK only interacted with the fusion protein containing HERC1's HECT domain. Top left: M2-PK bound to pulled-down GST fusion proteins. Bottom left: M2-PK levels in cell extracts. Right: Pulled-down GST fusion proteins. E: Glutathione-Sepharose beads bound to either GST or GST-HERC1 (aa 1–4861, full length) were incubated with a lysate from M2-PK baculovirus-infected Sf9 cells and beads were analyzed by SDS–PAGE/immunoblot. A small amount of M2-PK was specifically pulled-down by full-length HERC1.

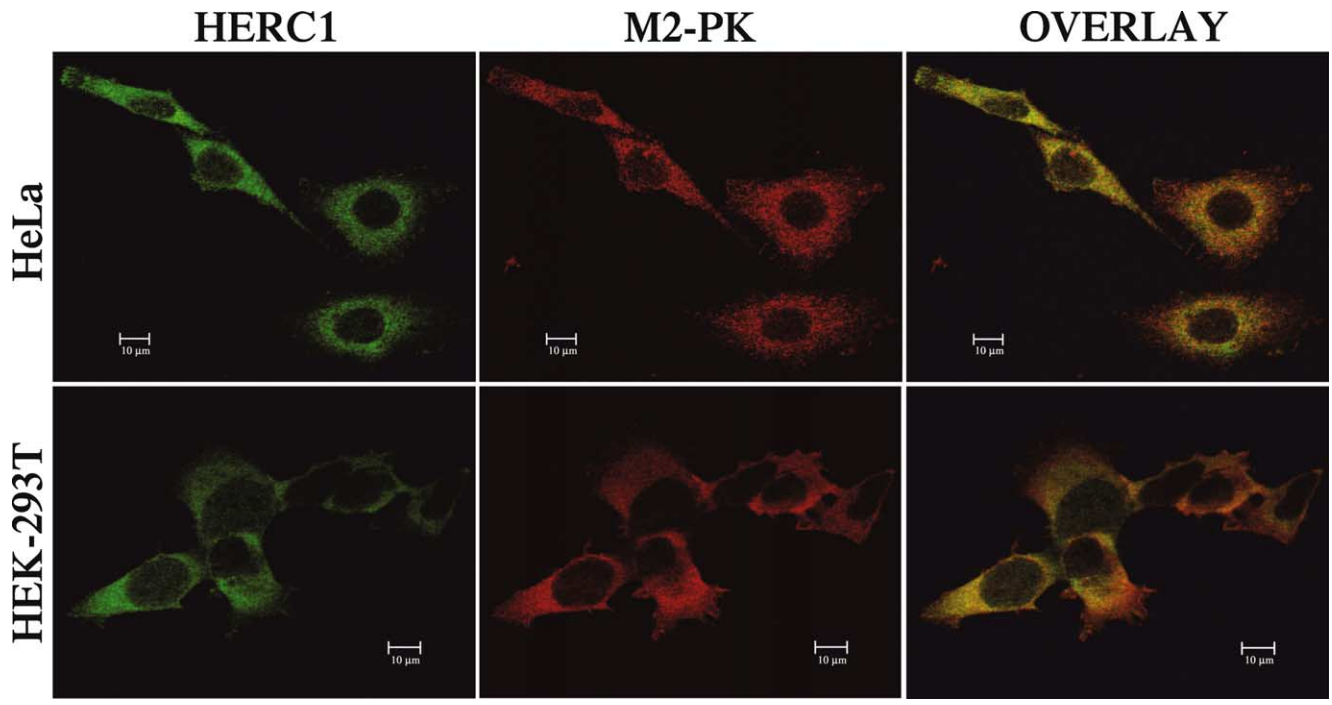

Fig. 2. Localization of endogenous HERC1 and M2-PK proteins was analyzed by confocal immunofluorescence microscopy in HeLa and HEK-293T cell lines. Both proteins display a perinuclear, punctate staining that presumably indicates their association with intracellular membranous structures. HERC1 and M2-PK show a partial colocalization in both cell lines when images are overlaid. All images correspond to one single cellular plane.

overexpressing HEK-293T cells (immunoblot analysis showed a 2–3-fold overexpression, data not shown). As shown in Fig. 3C, no significant changes were detected. Moreover, since HERC1 is known to form a thioester bond with ubiquitin in the presence of UbcH5 [17], we also checked whether the latter protein was necessary for an effect to take place. Nevertheless, addition of purified His-UbcH5a to the former extracts had no effect whatsoever on M2-PK enzyme activity (Fig. 3C). We also wondered whether HERC1 might have an effect upon M2-PK's dimer-to-tetramer ratio, as do other proteins such as papillomaviral protein E7 [19]. To address this issue, we loaded control, His-HECT-expressing and HERC1-overexpressing HEK-293T lysates into a gel filtration chromatography column in order to separate dimeric and tetrameric M2-PK. Elution profiles did not show any significant differences between those samples (data not shown). In summary, all these results appear to indicate that HERC1 does not affect M2-PK enzyme activity.

4. Discussion

In the present study, we have shown a physical interaction between HERC1, a protein involved in intracellular membrane traffic, and glycolytic isoenzyme M2-PK. This interaction was first found in the yeast two-hybrid system and it was shown to take place between the last 366 amino acid residues of HERC1, which encompass its HECT domain, and the last 126 residues of M2-PK including critical residues involved in fructose-1,6-bisphosphate binding and intersubunit contact (see below). The interaction was subsequently demonstrated to be direct by in vitro pull-down assays carried out with purified proteins and to occur in vivo in Sf9 insect cells when both full-length M2-PK and the last 366 or 1178 amino acid residues of HERC1 were expressed using recombinant baculoviruses. An interaction between both full-length proteins, albeit weak, could also be demonstrated in pull-down experiments in Sf9 cells. Moreover, human endogenous HERC1 and M2-PK proteins were shown to display similar, partially overlapping, perinuclear, punctate stainings when their subcellular localization was analyzed by immunofluorescence microscopy. This presumably indicates that both proteins are associated with intracellular membranous compartments [18] and is in agreement with previous data reporting M2-PK activation by phosphatidylserine-containing liposomes [20] and HERC1 localization in inner cell membranes [6]. However, in spite of all this evidence, we have failed to pinpoint the HERC1–M2-PK interaction in mammalian cells. Several reasons might explain this. One of them is the lack of good commercial antibodies to immunoprecipitate endogenous M2-PK or HERC1 proteins. Another possible reason may have to do with the low levels of expression achieved when full-length tagged HERC1 (His-HERC1 or Myc-HERC1) was transfected into mammalian cell lines (HeLa, HEK-293T, COS-1), which in turn is likely to be due to HERC1's giant size (4861 aa). Interestingly, we also could not find the interaction by immunoprecipitation of Myc-HERC1 (aa 3684–4861) or by pull-down of His-HERC1 (aa 4496–4861) in mammalian cells. In our opinion, these data probably indicate that M2-PK binding to HERC1 is tightly regulated in mammals and only happens under very specific conditions.

M2-PK undergoes a very complex regulation. Since it catalyzes the last step in glycolysis, the regulation of its activity is essential for cells to control the amount of carbon channeled into energy production (anaerobic glycolysis or the TCA cycle) and into biosynthetic pathways (M2-PK inhibition in-

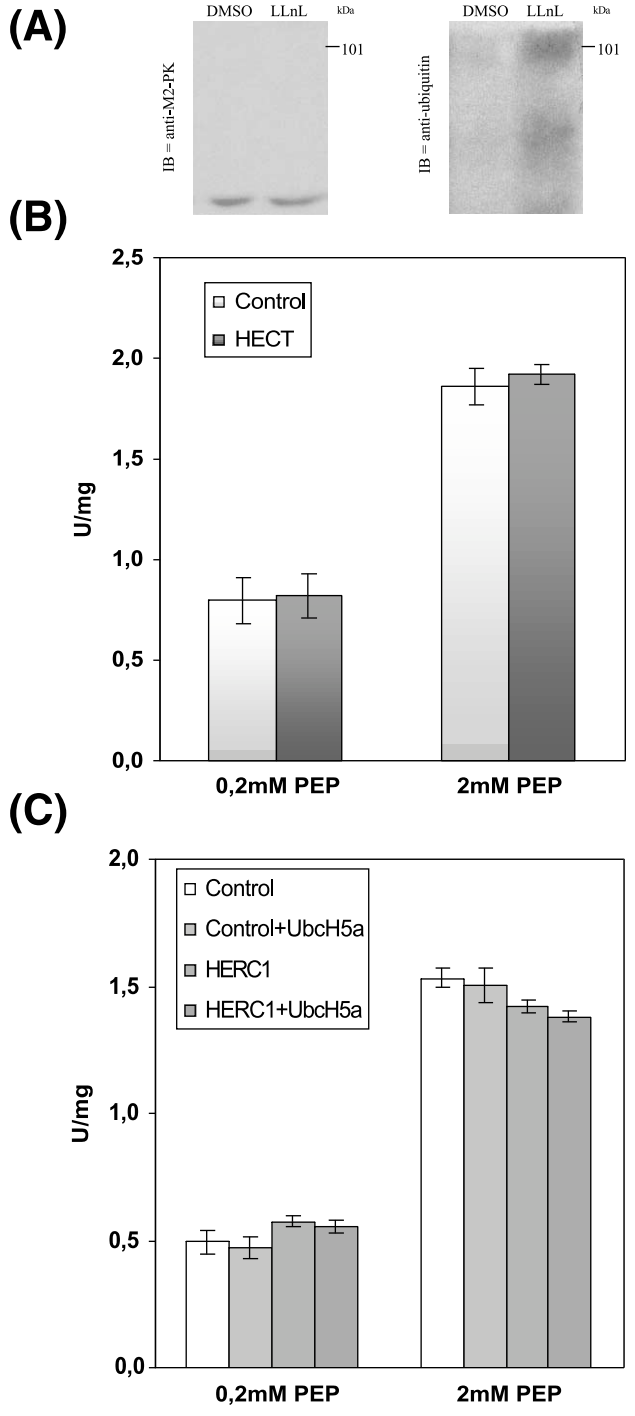

Fig. 3. A: Proteasome inhibitor LLnL does not affect M2-PK levels in HEK-293T cells. Immunoblot analysis showed that while other proteins became mono- or polyubiquitinated as a result of LLnL treatment, M2-PK did not undergo this modification nor were its protein levels significantly affected. B and C: M2-PK activity in HEK-293T cells is not affected by HERC1. B: Activity was analyzed in cells transfected with either control plasmid or a plasmid expressing His-tagged HERC1's HECT domain. C: Activity was analyzed in cells transfected with either control plasmid or a plasmid expressing full-length His-HERC1 (pFG3) in the absence or presence of UbcH5a. Activity was measured at two different phosphoenolpyruvate concentrations and has been expressed as units per mg of protein in the extracts (mean ± S.E.M. of six independent experiments).

duces an accumulation of glycolytic phosphometabolites that may act as biosynthetic precursors [21]). As a matter of fact, M2-PK is only one of four pyruvate kinase isoenzymes in mammals, the other three being L-PK, R-PK and M1-PK. The first two are expressed in liver and red blood cells, respectively, and arise from a single gene (the PKL gene) by differential promoter use. M1-PK, on the other hand, is mainly expressed in muscle and brain and is characterized by its hyperbolic Michaelis–Menten kinetics, in opposition to all other isoforms, which display sigmoidal kinetics. M1- and M2-PK are also synthesized from a single common gene (the PKM gene), which by differential splicing gives rise to the two proteins (these differ only in a short stretch of 56 amino acid residues corresponding to the alternatively spliced exon) [22,23]. M2-PK, like L-PK and R-PK, is allosterically regulated via feed-forward activation by fructose-1,6-bisphosphate (FBP) and via feedback inhibition by adenosine triphosphate (ATP) [21]. Furthermore, M2-PK can be found in cells in two major forms, namely, a highly active tetramer and a less active dimer. The equilibrium between these two forms can be shifted towards one or the other side by several factors: thus, while FBP induces tetramer formation, tyrosine phosphorylation or the E7 oncoprotein of carcinogenic human papillomaviruses inactivate the enzyme by converting it into the dimeric form [21,24]. All these regulatory properties, especially its ability to fine-tune the relative levels of energy production and biosynthesis, appear to make M2-PK the most suitable PK isoform for proliferating cells, which have high energetic and biosynthetic requirements at the same time. This would explain why M2-PK is expressed in all tissues during development and also why it is re-expressed when cells de-differentiate to become malignant or, still, why it is targeted by viral oncoproteins [19,23,24]. In fact, dimeric M2-PK, also known as tumor M2-PK, accumulates in malignant cells and has even turned out to be a good diagnostic marker for a number of cancers [25].

Any of these processes affecting M2-PK function could be regulating its interaction with HERC1. In this regard, we checked whether binding could be dependent on M2-PK being phosphorylated in tyrosine residues, which is known to inactivate the enzyme and could also shed some light on a possible role of HERC1 in tumorigenesis suggested by the fact that this protein is overexpressed in tumor cell lines [6]. Nonetheless, neither expression in HEK-293T cells of constitutively active Src (v-Src is known to be involved in tyrosine phosphorylation of M2-PK in chicken embryos [24]) nor epidermal growth factor treatment of A431 cells induced the association of both proteins (data not shown). It might also be that the interaction is somehow controlled by FBP levels, but this hypothesis, which is all the more reasonable in view of the fact that the HERC1 binding region in M2-PK includes many critical residues for FBP binding and intersubunit contact [26–28], still remains to be explored, as is the case for a possible role of the association of M2-PK to intracellular membranes.

Finally, we attempted to find a possible physiological significance for the interaction. In view of all available information about these proteins, it is reasonable to think that HERC1 could function as an E3 ubiquitin-protein ligase for M2-PK and/or modulate M2-PK enzyme activity. We tested both possibilities. First, since we were not able to find any ubiquitinated forms of M2-PK nor an increase in its expression levels in response to a proteasome inhibitor, we con-

cluded that M2-PK could not be a ubiquitination substrate of HERC1. On the other hand, overexpression of HERC1 or its HECT domain did not affect PK activity in HEK-293T cell lysates, nor did it alter its dimer-to-tetramer ratio. This activity is due only or almost only to the M2-PK isoform, since HEK-293T is an immortalized cell line [23]. Further insight into the functions of both HERC1 and M2-PK will still have to be gained before the physiological relevance of their interaction can be elucidated.

Acknowledgements: We thank Esther Adanero, Julieta Díaz, David Fernández, Miriam Villanueva and Bianca Kulik for technical assistance and Elena González for helpful discussion. Likewise, we are grateful to both Dr. Tamio Noguchi and Dr. Kazuhiro Iwai for their gifts of the M2-PK baculovirus and plasmid pT7-7-His-UbcH5a, respectively. This work was supported by grants from Ministerio de Ciencia y Tecnología (BMC2001-3744) and Generalitat de Catalunya (99SGR-0073). F.R.G.-G. has been supported by a doctoral fellowship (1999-FI-00246) from Generalitat de Catalunya.

References

[1] Scheffner, M., Huibregtse, J.M., Vierstra, R.D. and Howley, P.M. (1993) Cell 75, 495–505.
[2] Huibregtse, J.M., Scheffner, M., Beaudenon, S. and Howley, P.M. (1995) Proc. Natl. Acad. Sci. USA 92, 2563–2567.
[3] Huang, L., Kinnucan, E., Wang, G., Beaudenon, S., Howley, P.M., Huibregtse, J.M. and Pavletich, N.P. (1999) Science 286, 1321–1326.
[4] Renault, L., Kuhlmann, J., Henkel, A. and Wittinghofer, A. (2001) Cell 105, 245–255.
[5] Clarke, P.R. and Zhang, C. (2001) Trends Cell Biol. 11, 366–371.
[6] Rosa, J.L., Casaroli-Marano, R.P., Buckler, A.J., Vilaró, S. and Barbacid, M. (1996) EMBO J. 15, 4262–4273 (Corrigendum: EMBO J. 15, 5738).
[7] Cruz, C., Paladugu, A., Ventura, F., Bartrons, R., Aldaz, M. and Rosa, J.L. (1999) Cytogenet. Cell Genet. 86, 68–69.
[8] Cruz, C., Nadal, M., Ventura, F., Bartrons, R., Estivill, X. and Rosa, J.L. (1999) Cytogenet. Cell Genet. 87, 263–264.
[9] Lehman, A.L., Nakatsu, Y., Ching, A., Bronson, R.T., Oakey, R.J., Keiper-Hrynko, N., Finger, J.N., Durham-Pierre, D., Horton, D.B., Newton, J.M., Lyon, M.F. and Brilliant, M.H. (1998) Proc. Natl. Acad. Sci. USA 95, 9436–9441.
[10] Ji, Y., Walkowicz, M.J., Buiting, K., Johnson, D.K., Tarvin, R.E., Rinchik, E.M., Horsthemke, B., Stubbs, L. and Nicholls, R.D. (1999) Hum. Mol. Genet. 8, 533–542.
[11] Cruz, C., Ventura, F., Bartrons, R. and Rosa, J.L. (2001) FEBS Lett. 488, 74–80.
[12] Mitsui, K., Nakanishi, M., Ohtsuka, S., Norwood, T.H., Okabayashi, K., Miyamoto, C., Tanaka, K., Yoshimura, A. and Ohtsubo, M. (1999) Biochem. Biophys. Res. Commun. 266, 115–122.
[13] Rosa, J.L. and Barbacid, M. (1997) Oncogene 15, 1–6.
[14] Ponting, C., Schultz, J. and Borg, P. (1997) Trends Biochem. Sci. 6, 193–194.
[15] Cenciarelli, C., Chiaur, D.S., Guardavaccaro, D., Parks, W., Vidal, M. and Pagano, M. (1999) Curr. Biol. 20, 1177–1179.
[16] Jackson, P.K. and Eldridge, A.G. (2002) Mol. Cell 5, 923–925.
[17] Schwarz, S.E., Rosa, J.L. and Scheffner, M. (1998) J. Biol. Chem. 273, 12148–12154.
[18] Wasiak, S., Legendre-Guillemin, V., Puertollano, R., Blondeau, F., Girard, M., De Heuvel, E., Boismenu, D., Bell, A.W., Bonifacino, J.S. and McPherson, P.S. (2002) J. Cell Biol. 158, 855–862.
[19] Zwerschke, W., Mazurek, S., Massimi, P., Banks, L., Eigenbrodt, E. and Jansen-Dürr, P. (1999) Proc. Natl. Acad. Sci. USA 96, 1291–1296.
[20] Dabrowska, A., Pietkiewicz, J., Dabrowska, K., Czapinska, E. and Danielewicz, R. (1998) Biochim. Biophys. Acta 1383, 123–129.
[21] Mazurek, S., Grimm, H., Boschek, C.B., Vaupel, P. and Eigenbrodt, E. (2002) Br. J. Nutr. 87 (Suppl. 1), S23–29.
[22] Yamada, K. and Noguchi, T. (1999) Biochem. J. 337, 1–11.
[23] Yamada, K. and Noguchi, T. (1999) Biochem. Biophys. Res. Commun. 256, 257–262.
[24] Presek, P., Reinacher, M. and Eigenbrodt, E. (1988) FEBS Lett. 242, 194–198.
[25] Mazurek, S., Grimm, H., Oehmke, M., Weisse, G., Teigelkamp, S. and Eigenbrodt, E. (2000) Anticancer Res. 20, 5151–5154.
[26] Jurica, M.S., Mesecar, A., Heath, P.J., Shi, W., Nowak, T. and Stoddard, B.L. (1998) Structure 6, 195–210.
[27] Ikeda, Y. and Noguchi, T. (1998) J. Biol. Chem. 273, 12227–12233.
[28] Ikeda, Y., Taniguchi, N. and Noguchi, T. (2000) J. Biol. Chem. 275, 9150–9156.