THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 279, No. 13, Issue of March 26, pp. 12804–12811, 2004

Printed in U.S.A.

Comprehensive Proteomic Analysis of Human Par Protein Complexes Reveals an Interconnected Protein Network*[S]

Received for publication, November 6, 2003, and in revised form, December 8, 2003
Published, JBC Papers in Press, December 15, 2003, DOI 10.1074/jbc.M312171200

Miro Brajenovic, Gerard Joberty, Bernhard Küster, Tewis Bouwmeester‡, and Gerard Drewes‡

From Cellzome AG, Meyerhofstrasse 1, D-69117 Heidelberg, Germany

The polarization of eukaryotic cells is controlled by the concerted activities of asymmetrically localized proteins. The PAR proteins, first identified in *Caenorhabditis elegans*, are common regulators of cell polarity conserved from nematode and flies to man. However, little is known about the molecular mechanisms by which these proteins and protein complexes establish cell polarity in mammals. We have mapped multiprotein complexes formed around the putative human Par orthologs MARK4 (microtubule-associated protein/microtubule affinity-regulating kinase 4) (Par-1), Par-3, LKB1 (Par-4), 14-3-3ζ and η (Par-5), Par-6a, -b, -c, and PKCλ (PKC3). We employed a proteomic approach comprising tandem affinity purification (TAP) of protein complexes from cultured cells and protein sequencing by tandem mass spectrometry. From these data we constructed a highly interconnected protein network consisting of three core complex "modules" formed around MARK4 (Par-1), Par-3·Par-6, and LKB1 (Par-4). The network confirms most previously reported interactions. In addition we identified more than 50 novel interactors, some of which, like the 14-3-3 phospho-protein scaffolds, occur in more than one distinct complex. We demonstrate that the complex formation between LKB1·Par-4, PAPK, and Mo25 results in the translocation of LKB1 from the nucleus to the cytoplasm and to tight junctions and show that the LKB1 complex may activate MARKs, which are known to introduce 14-3-3 binding sites into several substrates. Our findings suggest co-regulation and/or signaling events between the distinct Par complexes and provide a basis for further elucidation of the molecular mechanisms that govern cell polarity.

Cell polarity is fundamental for the directional transport and positioning of vesicles and organelles, enabling the establishment of the embryonic body axis and cell fate decisions as well as maintaining fully functional differentiated cells (1–3). On the molecular level, the polarization of eukaryotic cells is enabled by the concerted activity of asymmetrically localized proteins and protein complexes (4).

The analysis of *Caenorhabditis elegans* mutants defective in asymmetric cell division has led to the identification of six "partitioning defective" (*PAR*)[1] genes (5–8). PAR proteins cooperate to convert transient polarity cues into a stable polarized cellular axis by influencing diverse processes like cytoskeletal dynamics or protein degradation (9, 10). At the one-cell stage, all PAR proteins except PAR-4 exhibit asymmetric localization to the opposite cortices of cells, and a functional hierarchy is inferred from the effect of mutants in each *PAR* gene on the localization of the other PAR proteins (7, 10–13). The 14-3-3 protein PAR-5 is at the top of this hierarchy, because it is required for the anterior localization of PDZ-domain proteins PAR-3 and PAR-6, which form a complex with PKC3, as well as for the posterior localization of PAR-2 and PAR-1. PAR-1 is at the base, because par-1 mutants have no effect on the localization of other PARs.

Whereas nematode PAR-2 has no obvious homolog in other organisms, the other PARs have putative orthologs. However their regulatory hierarchy seems to differ between organisms. In *Drosophila*, the localization of the PAR-3/PAR-6/PKC complex members is not co-dependent, nor are they, or PAR-5/14-3-3, required for the localization of PAR-1 during oogenesis (14, 15). Later, in the oocyte, PAR-1 becomes dependent on PAR-3 (16). There is evidence that PAR-5/14-3-3 may also function downstream of PAR-1 (15). Overexpression of *Drosophila* PAR-4/LKB1 partially rescues the par-1 phenotype, suggesting that the two kinases may function in a linear pathway (17).

In mammals, Par-3 and Par-6 have been shown to control polarity in a variety of cell types. Par-6 forms the core of a polarity complex due to its direct interaction with Par-3, aPKC (λ or ζ), the GTPases Cdc42 or Rac, as well as with the tumor suppressor mLgl (18–22). In epithelial cells, the binding of Par-3 and mLgl to Par-6·aPKC has been shown to be mutually exclusive resulting in opposite functions: Par-6 complexes containing Par-3 promote the formation of tight junctions, whereas complexes containing Lgl suppress them (21–23). Par-6 may also be involved in the regulation of tight junctions by interacting with components of the crumbs complex (24). The role of the Par-6 complex has also been studied in other polarized cell types. In astrocytes, Par-6·aPKCζ is part of a pathway starting from integrins and the subsequent activation of Cdc42, leading to the control of migration. Both Par-6 and aPKC bind directly to glycogen synthase kinase 3β and subsequently regulate the binding of the adenomatous polyposis coli protein to microtubules leading to major reorganization of the cell, whereas Par-3 is not required (25, 26). However, in differentiating neurons, the Par-3·Par-6·aPKC complex is crucial for determining which neurite is selected to become the axon (27).

* This work contains part of the doctoral thesis of M. B. at the University of Heidelberg. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[S] The on-line version of this article (available at http://www.jbc.org) contains Table 1 and Figs. S1, S2, and S3.

‡ To whom correspondence may be addressed. Tel.: 49-62-21-1375-7350; Fax: 49-62-21-1375-7206; E-mail: Tewis.Bouwmeester@Cellzome.com (T. B.) or Gerard.Drewes@Cellzome.com (G. D.).

[1] The abbreviations used are: PAR, partitioning-defective; PKC, protein kinase C; aPKC, atypical PKC; DTT, dithiothreitol; HA, hemagglutinin; HEK, human embryonic kidney; LC-MS/MS, liquid chromatography-tandem mass spectrometry; MAP, microtubule-associated protein; MARK, MAP/microtubule affinity-regulating kinase; MDCK, Madin-Darby canine kidney; TAP, tandem-affinity purification; ORF, open reading frame; PAPK, polyploidy-associated protein kinase; STRAD, STE20-related adaptor.

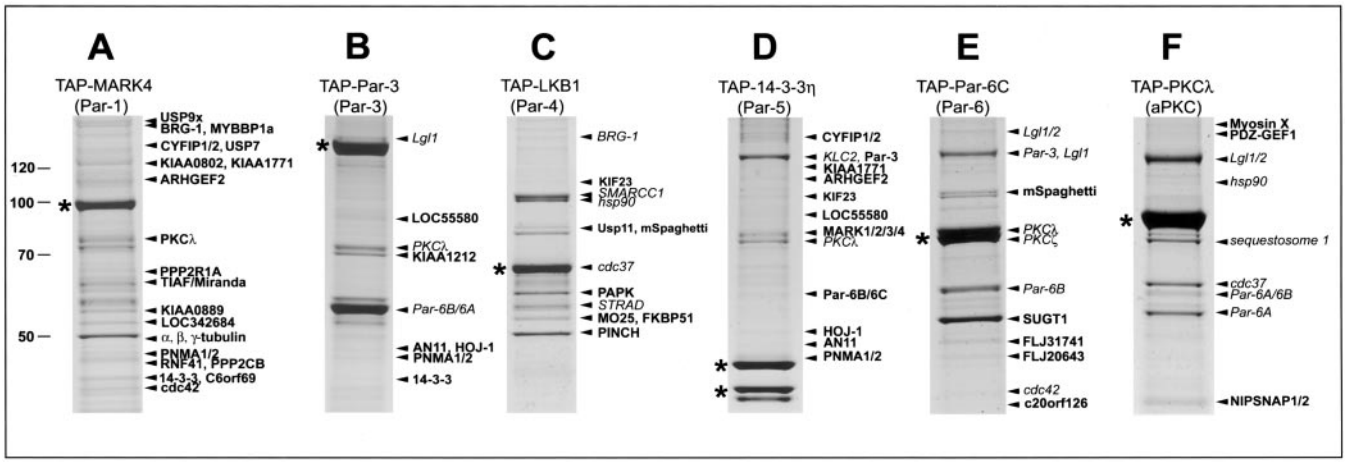


FIG. 1. **TAP purification of Par complexes.** Protein complexes formed by TAP-MARK4 (a homolog of *C. elegans* PAR-1), TAP-Par-3, TAP-LKB1 (the homolog of *C. elegans* PAR-4), TAP-14-3-3η (a homolog of *C. elegans* PAR-4), TAP-Par-6C, and TAP-PKCλ (the homolog of *C. elegans* PKC3) were purified from HEK293 cells, separated by SDS-PAGE, and stained with colloidal Coomassie. The tagged proteins are indicated with an *asterisk*. Co-purifying proteins identified by LC-MS/MS are labeled in *italics* (previously reported interactors) or *bold* (novel interactors), respectively.

The MARK kinases, which represent the mammalian homologs of PAR-1, were originally identified by their ability to phosphorylate microtubule-associated proteins (MAPs), and thus to regulate microtubule stability in cultured cells (28, 29). MARKs were also shown to be necessary for the establishment of polarity in cultured epithelial and neuroblastoma cells (30, 31). The human ortholog of PAR-4 is the serine/threonine kinase LKB1. Mutations in *LKB1* cause Peutz-Jeghers syndrome, an autosomal-dominant disorder characterized by gastrointestinal polyps and an increased risk of neoplasms, indicating that LKB1 is a tumor suppressor (32). Failure to establish cell polarity may result in inappropriate overgrowth of differentiated cells and cause neoplasms (33). Finally, the putative human orthologs of PAR-5, the 14-3-3 family proteins, have like LKB1 not been directly implied in cell polarity, but they are known to exert pleiotropic functions in processes crucial to polarity, *e.g.* vesicle trafficking (34).

It is not clear whether, in mammalian cells, the PAR proteins operate in a linear hierarchy similar to *C. elegans* and how they cooperate with other factors involved in polarity. Also it is not clear whether the orthologs of PAR-1 and PAR-4 operate in a protein complex similar to Par-3·Par-6 and how the "cross-talk" between the different polarity complexes is accomplished. In the present study, we employed tandem affinity purification (TAP) combined with liquid chromatography/tandem mass spectrometry (LC-MS/MS) to map protein complexes around human PAR orthologs. We show that all PAR orthologs form protein complexes and that these complexes share common interactors. Our data allow the construction of a network connecting known and novel players in cell polarity and offer new insight into the molecular mechanisms of morphogenesis.

EXPERIMENTAL PROCEDURES

cDNA Cloning—Expression vectors were generated by site-specific recombination (Gateway system, Invitrogen) of PCR-amplified ORFs into modified TAP-, HA-, or myc-tagged versions of the Moloney murine leukemia virus-based vector pZome1 (Cellzome; available from Euroscarf GmbH). For MARK4, LKB1, Par-6, aPKC, 14-3-3, and PNMA1, the TAP cassette was fused to the N terminus and for Par-3 to the C terminus. The inserted ORFs were subcloned from the following human MGC cDNA clones: aPKC, Image (National Institutes of Health): 4823853; 14-3-3ζ, Image: 2988020; 14-3-3η, Image: 3543571; LKB1, Image: 3689780; PNMA1, Image: 4431875; MARK4, Image: 3874322; MARK2, Image: 3139103; PAPK, Image: 3546243; CDC42 Image: 3626647; Mo25, Image: 4397573; Par-3, Image: 3939370; and Par-6C, Image: 4844008 (RZPD, Berlin, Germany). The Par-6a and b cDNAs were a kind gift of Dr. Ian Macara.

Cell Culture—Human embryonic kidney cells (HEK293, ATCC) were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Madin-Darby canine kidney (MDCK, ATCC) cells were maintained in minimal essential medium supplemented with 10% fetal calf serum and 2 mM glutamine. Pools of cells expressing epitope-tagged proteins were generated by retroviral gene transfer (35). For transient expression, ORFs were fused N-terminally with HA- or myc-epitopes (36). For microscopy, cells were grown on coverslips at 37 °C and 5% CO_2 in a humidified chamber. Plasmids (300 ng) were transfected into cells grown at 70% confluency using lipofectamin2000 (Invitrogen) according to the manufacturer's instructions. Cells were fixed either with methanol for 10 min at −20 °C or with 4% (v/v) paraformaldehyde for 15 min at 37 °C. After fixation, cells were blocked with 1% bovine serum albumin/0.1% Triton X-100 in phosphate-buffered saline and incubated for 1 h at 37 °C with polyclonal anti-HA (Abcam, UK) or monoclonal anti-myc (BD Clontech). Secondary antibodies were goat anti-mouse- or anti-rabbit-labeled with Oregon green or Texas red. DNA was stained with 4′,6-diamidino-2-phenylindole. Coverslips were mounted on glass slides with Vectashield (Vector Laboratories), and cells were imaged using a ×60 objective on an Olympus IX70 inverted microscope.

Immunoprecipitation—HEK293 cells were collected 48 h after transfection from one 10-cm dish in 400 μl of lysis buffer (250 mM Tris, pH 7.5, 25% glycerol, 7.5 mM $MgCl_2$, 0.5% (v/v) Triton X-100, 500 mM NaCl, 125 mM NaF, 5 mM Na_3VO_4, 1 mM DTT, Roche Applied Science protease inhibitor tablets). Lysates were cleared by centrifugation and used for immunoprecipitation with anti-myc (BD Clontech) or anti-HA resins (Roche Diagnostics, Mannheim, Germany) for 2 h at 4 °C. Immunoprecipitates were washed three times with lysis buffer and separated by SDS-PAGE. Proteins were transferred to nitrocellulose and detected using horseradish peroxidase-conjugated 3F10 or 9E10 at 1/10,000 (BD Clontech). For kinase assays, immunoprecipitates were washed three times with lysis buffer and once with kinase buffer (50 mM Hepes, pH 7.4, 10 mM $MgCl_2$, 200 μM ATP, 100 mM NaCl, 1 mM DTT). Beads were incubated with 1 μCi of [γ-^{32}P]ATP for 15 min at 30 °C, and the reaction was stopped with phosphoric acid. The samples were resolved on SDS-PAGE and/or spotted onto P81 paper, washed with 8% phosphoric acid, and analyzed by autoradiography. A specific peptide substrate, NVKSKIG**S**TENLK was used as substrate for MARK (28).

Tandem Affinity Purification—Cell lysates were incubated with 200 μl of IgG-agarose beads (Sigma) for 2 h at 4 °C (37). The beads were collected in 0.8-ml spin columns (MoBiTec) and washed with cleavage buffer (10 mM Tris, pH 7.5, 100 mM NaCl, 1 mM DTT, 0.1% Igepal, 0.5 mM EDTA). The beads were resuspended in cleavage buffer and incubated with 100 units of tobacco etch virus protease (Invitrogen) for 1 h at 16 °C. The eluate was transferred into a column containing 200 μl of calmodulin-agarose (Stratagene) in 10 mM Tris, pH 7.5, 100 mM NaCl, 1 mM DTT, 0.1% Igepal, 2 mM $MgCl_2$, 2 mM imidazole, 4 mM $CaCl_2$ and after washing with excess buffer the column was eluted with 600 μl of elution buffer (10 mM Tris, pH 8.0, 5 mM EGTA) at 37 °C. The freeze-dried eluates were separated on 4–12% NuPAGE gels (Novex) and stained with colloidal Coomassie.

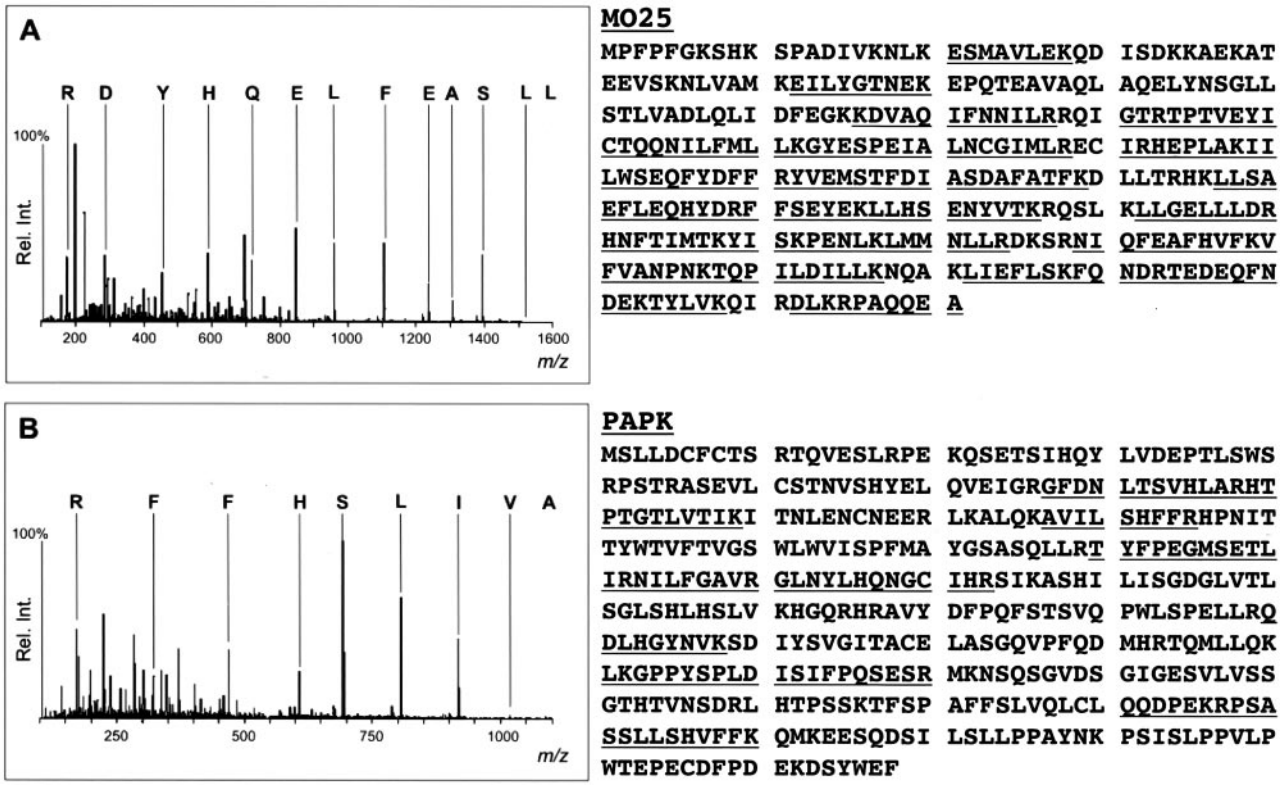


FIG. 2. **Examples of protein identifications: Two novel interactors of LKB1·Par-4.** *A*, LC-MS/MS spectrum of the tryptic peptide LLSAEFLEQHYDR from the Mo25 along with the underscored protein sequence showing all peptides that were sequenced from this protein. *B*, same for the tryptic peptide AVILSHFFR from PAPK.

Mass Spectrometry—Gels were sliced into 1.25-mm bands across the entire separation range of each lane to sample all potential interacting proteins without bias with respect to size and relative abundance. Cut bands were reduced, alkylated, and digested as described previously (38), and peptides were sequenced by tandem mass spectrometry (LC-MS/MS, QTOF Ultima and CapLC, Waters) (39). Proteins were identified by comparing the mass spectrometry sequence data against an in-house-curated version of the International Protein Index (available at www.ebi.ac.uk/IPI/) using the Mascot software package (Matrix Science, London, UK). Most proteins were unambiguously identified by the sequencing of several independent peptides (Supplemental Material, Table 1). For the few cases in which only one or two peptides supported the identification of a particular protein, we required these peptides to have a Mascot score indicating a $<5\%$ probability that the match could be considered a random event (40). In addition, these peptide matches were confirmed by using expressed sequence tags as an alternative search algorithm (41). A set of "sticky" proteins typically identified in the majority of TAP purifications, as judged by filtering against a control dataset consisting of more than 400 TAP purifications, were excluded from the analysis. This set includes heat shock proteins, ribosomal proteins, keratins, actin, myosin, and α- and β-tubulins. All experiments were carried out in duplicates.

RESULTS

Affinity Purification of Protein Complexes—Using retrovirus-mediated gene transfer, we created a panel of HEK293 cell pools expressing TAP-tagged fusions of nine human proteins representing putative orthologs of the *C. elegans* PAR gene products: MARK4 (*C. elegans* PAR-1), Par-3, LKB1 (*C. elegans* PAR-4), 14-3-3η, and ζ (*C. elegans* PAR−5), Par-6A, Par-6B, Par-6C, and PKCλ (*C. elegans* PKC3). TAP/MS analysis of the protein complexes formed around these nine proteins was performed from HEK293 cells, because we have assembled a data base that contains more than 5000 protein-protein interactions generated by TAP analysis in this cell line, which allows the reliable assessment of the specificity of a given interaction (data not shown). Expression levels were gauged by varying the multiplicity of infection and were typically 5–10 times over endogenous levels. Expanded cell pools were subjected to tandem affinity purification, a procedure consisting of two specific binding and two specific elution steps under mild conditions, which preserve the integrity of non-transient protein-protein interactions (37). The affinity-purified complexes were resolved on SDS-PAGE and Coomassie-stained (Fig. 1). The tagged bait proteins represent the most prominent bands on the gel, and in all cases distinct copurifying proteins are present (Fig. 1). Proteins were identified by peptide sequencing using tandem mass spectrometry (LC-MS/MS). Most proteins were identified by the sequencing of several peptides (Table 1). Details on protein identification procedures are given under "Experimental Procedures." As a representative example, Fig. 2 shows the identification of two interactors in the LKB1·Par-4 complex, which are identified by sequenced peptides covering 65% (Mo25) and 26% (PAPK) of their total sequence, respectively.

TAP Analysis of Par-3·Par-6·aPKC Complexes—We subjected several core members of the Par-6 polarity complex to TAP/MS analysis (Par-6C, Par-3, and aPKCλ (Fig. 1); Par-6A, Par-6B, and Fig. S1 in Supplemental Material). This complex has been studied extensively by yeast two-hybrid and co-immunoprecipitation analysis (19, 20), but to date a mapping of the polarity complex from mammalian cells by comprehensive tagging of the core scaffold proteins has not been reported. We detected the known components Par-6, Par-3, aPKC, and Lgl in all cases (Fig. 1). Cdc42 was detected only in the three TAP-Par-6 purifications and Rac was not found, presumably because the interaction of the small GTPases with Par-6 is transient. As previously reported, we found glycogen synthase kinase 3β to interact with all three Par-6 proteins, albeit only detectable by immunoblotting (data not shown) (26). Par-3 and Lgl are thought to bind competitively to the same site on Par-6 (21, 22). Nonetheless low amounts of Lgl were detected, judged by low sequence coverage, in the TAP-Par-3 purification. Although it

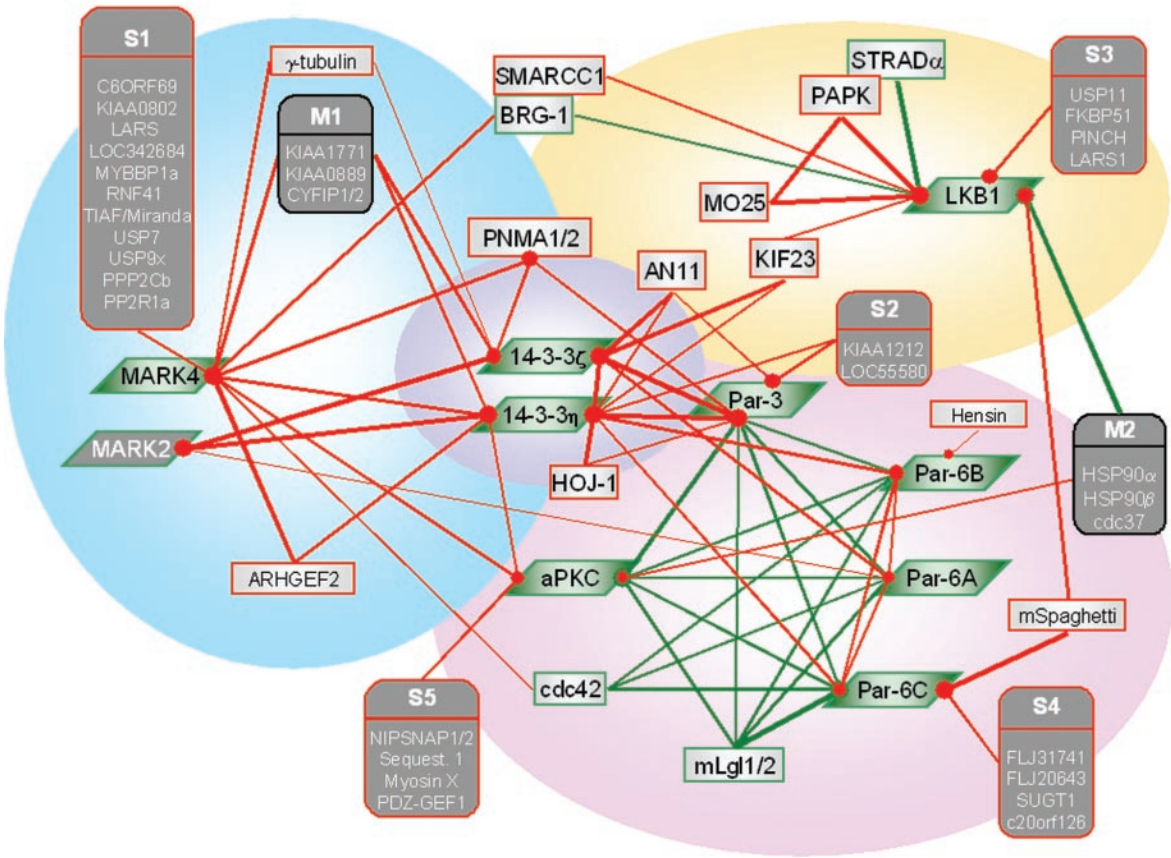
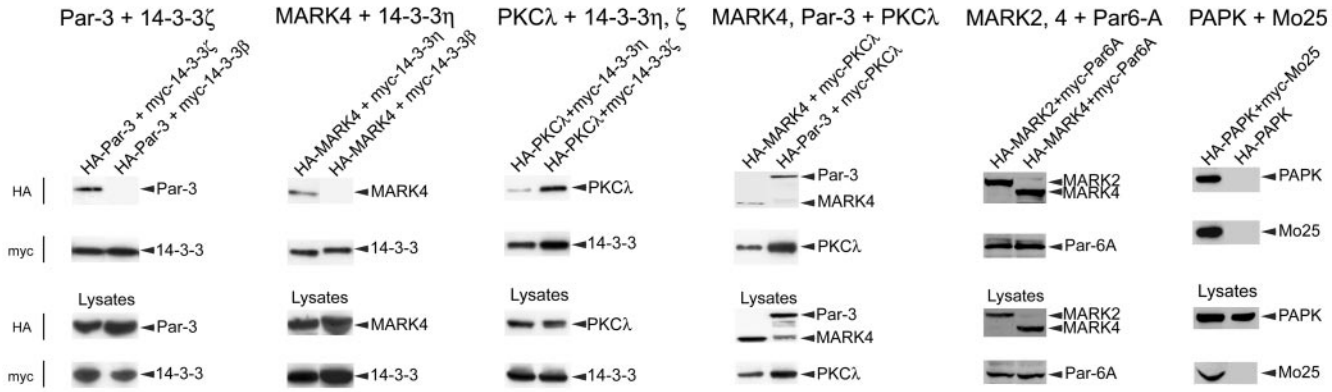


FIG. 3. **Connectivity map of human Par complexes based on TAP purifications and co-immunoprecipitation experiments.** The TAP-tagged proteins used as baits are represented as *rhomboids*. Lines connecting proteins indicate presence in a TAP complex or co-immunoprecipitation (*dotted lines*). The *width of each line* represents the degree of sequence coverage of the identification, which depends on the robustness of the interaction but also on the expression level and a number of other factors. *Green boxes/lines* represent previously known interactors/interactions; *red boxes/lines* represent novel interactors/interactions. Proteins that are found specifically with only one TAP-protein are grouped in *boxes* (*S1–S6*), whereas proteins that are consistently found together with more than one TAP-protein are grouped in modules (*M1* and *M2*).

has been previously reported that Lgl2 does not bind to Par-6C (22), we found it to be present abundantly in TAP-Par-6C. Notably, each Par-6 paralog was also found to be in complex with the two other paralogs. Because they do not bind directly to each other (19), this suggests the presence of higher order complexes. Several new interactors of the Par-6 polarity complex were identified (Table 1 and Figs. 1*E* and S1). Hensin, a large protein involved in the terminal differentiation of epithelia, was found in TAP-Par-6B (Fig. S1 in the Supplemental Material) and several proteins lacking functional annotation were found in TAP-Par-6C, including a protein related to *Drosophila* Spaghetti, a microfilament-binding protein involved in cytokinesis. In TAP-PKCλ, several novel interacting proteins were identified in addition to the known Sequestosome protein (42) (see Fig. 1*F* and Table 1). However, because these interactors were not found by tagging of the other polarity complex members, it is likely that they are involved in PKCλ functions distinct from the control of polarity. Unexpectedly, we found MARK2, another homolog of *C. elegans* PAR-1, in the TAP-Par-6A purification, and its paralog MARK4 was identified with TAP-aPKCλ, suggesting molecular cross-talk between the Par-6 polarity complexes and MARK·Par-1 complexes. We found several more components shared between the different polarity complexes: 14-3-3 proteins, which represent homologs of *C. elegans* PAR-5, were found with TAP-Par-3, and PNMA1, a marker for paraneoplastic syndrome, was identified with TAP-Par-3, TAP-14-3-3, and TAP-MARK4 (Fig. 1), but not with any other protein in our HEK293 dataset (data not shown). Reciprocally, we identified Par-3 and PKCλ in a TAP-PNMA1 complex (Fig. S2 in the Supplemental Material).

TAP Analysis of MARK4·Par-1 Complexes—The homologs of *C. elegans* PAR-1 are protein serine kinases conserved from yeast to man (28, 43). The mammalian MARK·Par-1 family comprises four closely related gene products. In flies, PAR-1 has been reported to interact with 14-3-3 proteins (15). This interaction is conserved, because we find several different 14-3-3 proteins in TAP-MARK4. 14-3-3 proteins are known to occur as homo- and heterodimers in cells and hence can serve as bridging factors. By co-immunoprecipitation it appears that MARK4 and MARK2 interact mainly with 14-3-3η, whereas Par-3 interacts mainly with 14-3-3ζ (see Fig. S1 in Supplementary Material). In total, the analysis of TAP-MARK4 resulted in the unambiguous identification of 20 interacting proteins (Fig. 1*A*). Among these, PKCλ, and cdc42 are components of the Par-6 polarity complex, and homologs of transforming growth factor-β-induced anti-apoptotic factor and 14-3-3 have been implicated in cell polarity. Transforming growth factor-β-induced anti-apoptotic factor may be an ortholog of Miranda, a protein that is associated with centrosomes and is involved in neuroblast asymmetric division in *Drosophila* (44). Interestingly MARK4 also associates with centrosomes and microtubules (45). Several interactors appear to be associated with the cytoskeleton; *e.g.* ARHGEF2, microtubule-associated exchange factor for Rac and Rho GTPases. We also find the microtubule-associated phosphatase 2A associated with TAP-MARK, which has been, like MARK·Par-1 itself, implicated in the regulation

A



B

	MARK4	Par-3	14-3-3	Par-6A	Par-6B	Par-6C	PKCλ	PNMA1	cdc42
MARK4 (Par-1)									
Par-3	0								
14-3-3 (Par-5)	2	2							
Par-6A	2	**2**	1						
Par-6B	0	**2**	1						
Par-6C	0	**2**	1						
PKCλ	1	**2**	1	**2**	**2**	**2**			
PNMA1	1	2	0	0	2	2	1		
cdc42	1	**0**	0	**2**	**2**	**2**	**2**	0	
LKB1 (Par-4)	1	1		0	0				

	LKB1	MO25
LKB1 (Par-4)		
MO25	2	
PAPK	2	2

FIG. 4. **Co-immunoprecipitation matrix of interactions between Par proteins and newly identified proteins.** HEK293 cells were transfected to express HA- and myc-tagged proteins as indicated, and proteins were immunoprecipitated using anti-myc antibodies and detected by immunoblot. *A*, examples of co-immunoprecipitation. Whole cell lysates are shown as expression control. *B*, summary of co-immunoprecipitation results: *0*, proteins not found to be associated; *1*, interaction seen only with one of the two proteins immunoprecipitated; *2*, proteins were found associated regardless of which protein was subjected to immunoprecipitation. All previously known interactions are shown in *boldface*.

of the microtubule-associated protein tau (46). Most of the other interacting proteins yet lack functional annotation.

In *C. elegans*, the function of PAR-1 is partially dependent on PAR-2, a RING finger protein with no obvious homolog in mammals. We found that MARK4 interacts with the RING finger protein RNF41. Despite the fact that PAR-2 and human RNF41 have no significant homology outside the RING domain, it is tempting to speculate that RNF41 may be functionally orthologous to PAR-2.

TAP Analysis of LKB1·Par-4 Complexes—In the TAP-LKB1 purification, we identified five known and seven novel interacting proteins. The pseudokinase STRAD was recently identified as a regulator of LKB1 (47). In addition to STRAD, we found a related protein PAPK, which is likely also a pseudokinase, as a prominent interactor of LKB1 (Table 1 and Figs. 1*C* and 2). We also identified Mo25, a protein with a conserved role in cell polarity in yeast and fly (48), hence representing the first interacting protein that links LKB1 to cell polarity. Mo25 and STRAD interact directly, because they quantitatively co-immunoprecipitate when transfected in HeLa cells, where LKB1 is not expressed (Fig. S3). Another interacting protein, the LIM domain protein PINCH, is involved in integrin-dependent cell adhesion and is essential for *Drosophila* development (49). Some of the other interacting proteins are shared with other Par complexes, such as BRG-1, which was also found with MARK4·Par-1, and FLJ21908/mSpaghetti with Par-6C, suggesting molecular cross-talk between the different complexes.

TAP Analysis of 14-3-3ζ and η—C. elegans PAR5 represents a homolog of the mammalian 14-3-3 family of proteins. TAP analysis of 14-3-3ζ and η yielded a plethora of interacting

proteins, including most of the known 14-3-3 interactors, *e.g.* kinesins, A-, B-, C-RAF, KSR, BAD, IRS1–2, and histone deacetylases, in addition to more than 100 novel 14-3-3 interacting proteins (data not shown). About half of these novel interactors found with TAP-14-3-3 contain the 14-3-3 recognition sequence motif, RS*X*(P_i)S*X*P (50) (data not shown). In this study we only focus on interacting proteins that link 14-3-3 to any of the other Par complexes. In TAP-14-3-3ζ and η we identified all four MARK kinases, confirming the results obtained with TAP-MARK4. We also find Par-3, Par-6a-c, and PKCλ, confirming the TAP-Par-3 results.

Par Protein Complexes Form a Highly Interconnected Network—The main results were depicted in a physical connectivity map around the four main complexes formed by Par-6·Par-3·aPKC, Par-1, Par-4, and Par-5 (Fig. 3). This network emphasizes the interconnection between the different Par protein clusters, which may suggest mechanisms of molecular cross-talk. However, it is important to note that a connection between two proteins in this network represents their presence in a complex, but does not necessarily imply a direct interaction.

We assessed some of these interesting new interactions by co-immunoprecipitation using HA- and myc-tagged proteins (Fig. 4). These data confirm the interaction between Par-6A and the Par-1 proteins MARK2 and MARK4, and the interaction between PKCλ and MARK4. In addition they show the specificity of binding of MARK4 and PNMA1 toward the Par-6 proteins: MARK4 interacts only with Par-6A, whereas PNMA1 interacts with Par-6B and Par-6C but not with Par-6A (Fig. 4*B*). All mutual interactions between LKB1, PAPK, and Mo25 were found by co-immunoprecipitation, suggesting a stable trimeric complex.

A Novel LKB1·Par-4 Shuttling Complex Containing PAPK and Mo25—To strengthen the physiological relevance of the new interactions identified with the TAP method, we analyzed the novel trimeric LKB1·Par-4 complex in a well polarized cell type, the MDCK epithelial cell line. Although LKB1 has been described as a predominantly nuclear protein, and its sequence contains a nuclear localization signal, a small but reproducible fraction of the protein has been detected in the cytoplasm, but it was not clear how this is regulated (51). When transduced separately at low levels of overexpression, both LKB1 and Mo25 show pronounced nuclear localization, with little cytoplasmic staining (Fig. 5, *top panels a* and *c*). PAPK is found in the cytoplasm, with prominent perinuclear staining (Fig. 5, *panel b*). Interestingly, co-overexpression of LKB1 and PAPK redistributes both proteins to the cytoplasm and to cell-cell contact areas (Fig. 5, *panels d–f*). This location partially overlaps with the tight junction belt as seen by co-localization with co-expressed Par-3 (Fig. 5, *panels g–i*) or co-staining of endogenous ZO-1 (data not shown.) In contrast, expression of Mo25 redistributes PAPK mainly into the nucleus, even though a small fraction of both proteins co-localizes at the plasma membrane (Fig. 5, *panels j–l*).

The pseudokinase STRAD has recently been shown to be a substrate of LKB1 (47). In an analogous fashion, it appears that PAPK is also a substrate of LKB1 when ectopically co-expressed in HEK293 cells. PAPK is phosphorylated by LKB1, and the level of LKB1 autophosphorylation was increased in presence of PAPK (Fig. 5, *bottom*).

In light of the fact that LKB1 redistributes to cell-cell contacts, where Par-1/MARK proteins also reside (30), and that some interacting proteins are shared between LKB1 and MARK4, we asked whether the two serine kinases may act in a linear pathway. In *Drosophila*, LKB1 can partially compensate the loss of PAR-1 (17). We did not find both kinases together in any purification, but the interaction may be transient or the expression level of endogenous LKB1 may be too low to be detected. However, when ectopically expressed in HEK cells,

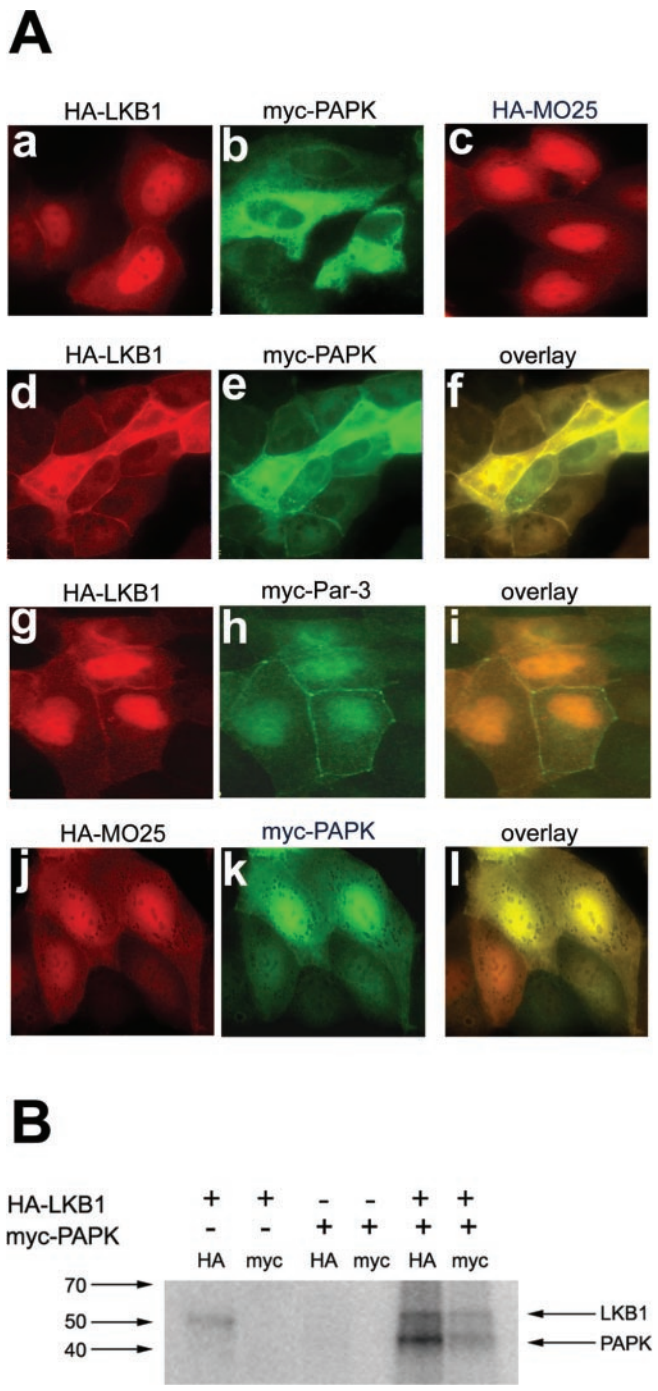


FIG. 5. **LKB1 is part of a Par-4 complex with Mo25 and PAPK, which is recruited to cell-cell contacts in a PAPK dependent fashion.** *A*, MDCK cells were transfected with HA-LKB1 (*a*), myc-PAPK (*b*), HA-Mo25 (*c*), HA-LKB1 plus myc-PAPK (*d–f*), HA-LKB1 plus myc-Par-3 plus PAPK (*g–i*), or HA-Mo25 plus myc-PAPK (*j–l*). Proteins were visualized by indirect immunofluorescence. LKB1 and Mo25 are mainly localized to the nucleus whereas PAPK is in the cytoplasm (*a–c*). Overexpression of PAPK recruits LKB1 to the cell-cell contacts (*d–f*) where Par-3 is located (*g–i*). Co-expression of Mo25 and PAPK redistributes both proteins to the nucleus, although a small fraction of the complex is recruited to the plasma membrane (*j–l*). *B*, PAPK is phosphorylated by LKB1 *in vitro* and increases LKB1 autophosphorylation. HA-LKB1 and myc-PAPK transfected into HEK293 cells were immunoprecipitated using either HA or myc antibodies as indicated. The immunoprecipitates were incubated with [γ-^{32}P]ATP and subjected to autoradiography and Western blotting as indicated.

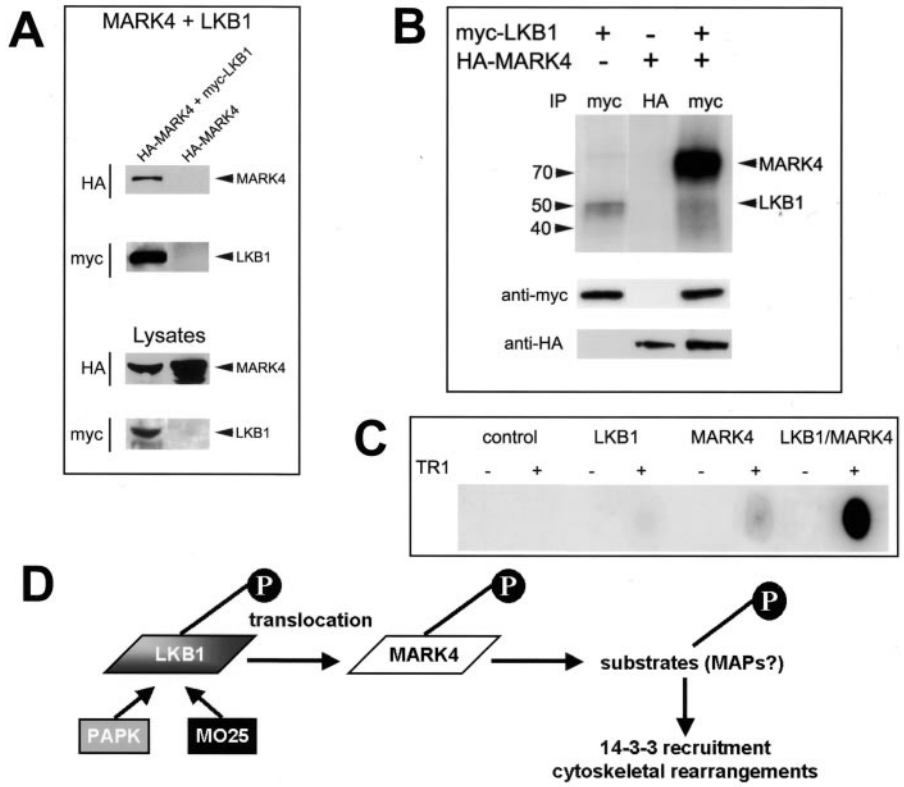


FIG. 6. **LKB1 binds to, phosphorylates, and activates MARK4.** *A*, HA-MARK4 and myc-LKB1 co-immunoprecipitate from HEK293 cells. *B*, HA-MARK4 and myc-LKB1 were expressed alone or together as indicated. Proteins were immunoprecipitated using anti-myc antibodies, incubated with [γ-^{32}P]ATP, and subjected to autoradiography and Western blotting as indicated. MARK4 and LKB1, when expressed alone, are phosphorylated to a low extent (*lanes 1* and *2*), but MARK4 is efficiently phosphorylated when co-expressed with LKB1 (*lane 3*). *C*, the immunoprecipitates shown under *B* were incubated with a MARK synthetic peptide substrate, which is not phosphorylated by LKB1. Samples were spotted on phosphocellulose paper and subjected to autoradiography. Co-expression with LKB1 dramatically stimulated MARK4 activity. *D*, diagram summarizing a hypothetical LKB-1-MARK signalling cascade. LKB-1 is activated by the recruitment of Mo25 and PAPK, and translocates to the periphery of the plasma membrane. Here it phosphorylates and activates MARK4, which in turn phosphorylates several substrates and creates 14-3-3 binding sites on them, which leads to downstream effects like cytoskeletal rearrangements.

both proteins interact (Fig. 6*A*), and MARK4 was strongly phosphorylated in presence of the LKB1·Par-4 complex (Fig. 6*B*), but we did not observe any increase of the phosphorylation level of LKB1. Hence we investigated whether the phosphorylation of MARK4 by LKB1 results in the activation of MARK4. Using a synthetic peptide as a specific substrate for MARK (28), we show that the activity of MARK4 is strongly enhanced in the presence of LKB1 (Fig. 6*C*). A similar activation by LKB1 was also observed with other MARKs (data not shown), implying MARK kinases downstream of LKB1 in a novel signaling pathway (Fig. 6*D*).

DISCUSSION

The current study represents the first comprehensive protein interaction analysis of Par proteins and aims to shed light on the regulatory molecular circuitry underlying the functional hierarchy of human Par orthologs. So far, most systematic protein-protein interaction studies and protein networks rely on yeast two-hybrid techniques (52). Compared with these approaches, affinity purification of complexes coupled to mass spectrometry-based protein identification offers the advantage of studying actual molecular assemblies made up by direct and cooperative interactions, which are purified from human cells under close to physiological conditions, rather than reconstituted bimolecular interactions *ex vivo* (53). Whereas in the yeast two-hybrid system both interactors are expressed as fusion proteins, in the TAP approach only one component is expressed as a fusion protein, and several components of one complex can be tagged to resolve different subcomplexes (53).

Using the TAP/MS approach, we have affinity-purified and mapped Par protein complexes from cultured cells, and identified the complex components by mass spectrometry (Fig. 1). From the data on the components of the purified complexes we constructed a comprehensive protein-protein interaction map around the human Par protein orthologs (Fig. 3). The map is built around nine bait proteins and contains a total of 60 proteins. Some of the novel interactors in our map have been previously implicated in the control of cell polarity, but not in connection with the Par proteins, but many of the novel interactors have not been previously implicated in cell polarity.

The network reproduces almost all of the published interactors of, and interactions between, the Par proteins. Some previously described interactors of the Par-6 complex were not found likely, because they are cell type-specific (24, 54). We found novel interacting proteins for most of the TAP baits, the majority being specific for their respective bait protein (boxes labeled *S1* to *S6* in Fig. 4). Some of the newly identified binders have already been linked directly or indirectly to cell polarity, *e.g.* Hensin, mSpaghetti, or Mo25. As mentioned above, several MARK·Par-1 interactors are possibly involved in cytoskeletal remodeling. For other interactors, it is more difficult to determine whether they are really part of a Par polarity complex. For example, some of the proteins involved in Par complexes, *e.g.* PKCλ, are likely to have additional cellular functions not relating to cell polarity, and some of these interactors may reflect such functions. Proteins that are found with two or more distinct Par complexes are, however, likely to play a role in the polarity network. An interesting example is represented by PNMA1 and PNMA2, which were found in TAP complexes of Par-3, MARK4, and 14-3-3ζ, respectively. However, the most prominent example of an interactor connecting several Par "modules" are the 14-3-3 proteins ζ and η, which form homo- and heterodimers and represent possible functional orthologs of *C. elegans* PAR-5. They interact with the Par-1 kinases MARK2 and MARK4, as well as with the adaptor protein Par-3. 14-3-3 proteins are phosphospecific adaptor proteins with multiple diverse functions, some related to cell polarity (7, 15). Par-3 bound to 14-3-3 appears not to interact with Par-6 or other members of the polarity complex (14-3-3s interact with Par-6s). It is tempting to speculate that the 14-3-3 interactors are regulated by phosphorylation by the MARK kinases, which are known to introduce 14-3-3 binding sites into their substrates (55, 56).

The human PAR-4 ortholog, LKB1, has until very recently not been implied in a multiprotein complex. We find that LKB1 forms a stable, predominantly cytoplasmic tri- or multimeric complex with the pseudokinases STRAD (47) or PAPK (57), and the adaptor protein Mo25, which is yet uncharacterized in mammals (48). It is possible that the nuclear localization signal in LKB1 is masked in the context of the complex. Co-expression of LKB1 and PAPK redistributes both proteins to cell-cell contact areas (Fig. 5, *panels d–f*). This location partially overlaps with the tight junction belt and is known to contain Par-1 family kinases like MARK2 and the Par-6 polarity complex (19, 30). We do not yet understand what the function of the LKB1·Par-4 complex in cell-cell contacts is, but we could show that LKB1·Par-4 can dramatically activate MARK4 (and other MARKs, data not shown) *in vitro*, which in turn may lead to the phosphorylation of substrates, *e.g.* microtubule-associated proteins, which in overexpression models have been shown to cause an increase in microtubule dynamics and alterations in cellular shape (28). This cascade may be involved in the reaction of neuronal cells to certain insults, because MARK4 has been implied in the hyperphosphorylation of tau protein in neurons affected by Alzheimer's disease (45), and it was recently found to be rapidly up-regulated in a mouse model of focal ischemia. Interestingly, LKB1 was also among the 56 genes that were found to be up-regulated in this model (58).

In summary, our interaction proteomics approach has provided a physical map of Par protein complexes demonstrating an unexpected degree of connectivity between the individual complexes. We have identified a large number of novel interactors, some of which interact with more than one Par complex. The fact that three of the central proteins in this network (Par-1, PKCλ, and Par-4) are protein serine kinases, taken together with the multiple connections of the 14-3-3 proteins ζ and η, in the physical map, tempts us to speculate that many of the molecular events by which Par proteins orchestrate cell polarity will be regulated by phosphorylation. The wealth of data provided in this study should provide a point of departure for more in depth studies that will eventually lead to the complete description of these events in molecular terms.

Acknowledgment—We thank all members of the Cellzome mass spectrometry group for outstanding technical expertise and diligence.

REFERENCES

1. Jan, Y. N., and Jan, L. Y. (1998) *Nature* **392,** 775–778
2. Mellman, I., and Warren, G. (2000) *Cell* **100,** 99–112
3. Keller, R. (2002) *Science* **298,** 1950–1954
4. Nelson, W. J. (2003) *Nature* **422,** 766–774
5. Kemphues, K. J., Priess, J. R., Morton, D. G., and Cheng, N. S. (1988) *Cell* **52,** 311–320
6. Watts, J. L., Etemad-Moghadam, B., Guo, S., Boyd, L., Draper, B. W., Mello, C. C., Priess, J. R., and Kemphues, K. J. (1996) *Development* **122,** 3133–3140
7. Morton, D. G., Shakes, D. C., Nugent, S., Dichoso, D., Wang, W., Golden, A., and Kemphues, K. J. (2002) *Dev. Biol.* **241,** 47–58
8. Tabuse, Y., Izumi, Y., Piano, F., Kemphues, K. J., Miwa, J., and Ohno, S. (1998) *Development* **125,** 3607–3614
9. Pellettieri, J., and Seydoux, G. (2002) *Science* **298,** 1946–1950
10. Cuenca, A. A., Schetter, A., Aceto, D., Kemphues, K., and Seydoux, G. (2003) *Development* **130,** 1255–1265
11. Kemphues, K. (2000) *Cell* **101,** 345–348
12. Etemad-Moghadam, B., Guo, S., and Kemphues, K. J. (1995) *Cell* **83,** 743–752
13. Guo, S., and Kemphues, K. J. (1995) *Cell* **81,** 611–620
14. Yamanaka, T., Horikoshi, Y., Suzuki, A., Sugiyama, Y., Kitamura, K., Maniwa, R., Nagai, Y., Yamashita, A., Hirose, T., Ishikawa, H., and Ohno, S. (2001) *Genes Cells* **6,** 721–731
15. Benton, R., Palacios, I. M., and St Johnston, D. (2002) *Dev Cell* **3,** 659–671
16. Vaccari, T., and Ephrussi, A. (2002) *Curr. Biol.* **12,** 1524–1528
17. Martin, S. G., and St Johnston, D. (2003) *Nature* **421,** 379–384
18. Qiu, R. G., Abo, A., and Martin, S. G. (2000) *Curr. Biol.* **10,** 697–707
19. Joberty, G., Petersen, C., Gao, L., and Macara, I. G. (2000) *Nat. Cell Biol.* **2,** 531–539
20. Lin, D., Edwards, A. S., Fawcett, J. P., Mbamalu, G., Scott, J. D., and Pawson, T. (2000) *Nat. Cell Biol.* **2,** 540–547
21. Plant, P. J., Fawcett, J. P., Lin, D. C., Holdorf, A. D., Binns, K., Kulkarni, S., and Pawson, T. (2003) *Nat. Cell Biol.* **5,** 301–308
22. Yamanaka, T., Horikoshi, Y., Sugiyama, Y., Ishiyama, C., Suzuki, A., Hirose, T., Iwamatsu, A., Shinohara, A., and Ohno, S. (2003) *Curr. Biol.* **13,** 734–743
23. Gao, L., Joberty, G., and Macara, I. G. (2002) *Curr. Biol.* **12,** 221–225
24. Hurd, T. W., Gao, L., Roh, M. H., Macara, I. G., and Margolis, B. (2003) *Nat. Cell Biol.* **5,** 137–142
25. Etienne-Manneville, S., and Hall, A. (2001) *Cell* **106,** 489–498
26. Etienne-Manneville, S., and Hall, A. (2003) *Nature* **421,** 753–756
27. Shi, S. H., Jan, L. Y., and Jan, Y. N. (2003) *Cell* **112,** 63–75
28. Drewes, G., Ebneth, A., Preuss, U., Mandelkow, E. M., and Mandelkow, E. (1997) *Cell* **89,** 297–308
29. Drewes, G., Trinczek, B., Illenberger, S., Biernat, J., Schmitt-Ulms, G., Meyer, H. E., Mandelkow, E. M., and Mandelkow, E. (1995) *J. Biol. Chem.* **270,** 7679–7688
30. Bohm, H., Brinkmann, V., Drab, M., Henske, A., and Kurzchalia, T. V. (1997) *Curr. Biol.* **7,** 603–606
31. Brown, A. J., Hutchings, C., Burke, J. F., and Mayne, L. V. (1999) *Mol. Cell Neurosci.* **13,** 119–130
32. Jenne, D. E., Reimann, H., Nezu, J., Friedel, W., Loff, S., Jeschke, R., Muller, O., Back, W., and Zimmer, M. (1998) *Nat. Genet.* **18,** 38–43
33. Bardeesy, N., Sinha, M., Hezel, A. F., Signoretti, S., Hathaway, N. A., Sharpless, N. E., Loda, M., Carrasco, D. R., and DePinho, R. A. (2002) *Nature* **419,** 162–167
34. O'Kelly, I., Butler, M. H., Zilberberg, N., and Goldstein, S. A. (2002) *Cell* **111,** 577–588
35. Morgenstern, J. P., and Land, H. (1990) *Nucleic Acids Res.* **18,** 3587–3596
36. Jarvik, J. W., and Telmer, C. A. (1998) *Annu. Rev. Genet.* **32,** 601–618
37. Rigaut, G., Shevchenko, A., Rutz, B., Wilm, M., Mann, M., and Seraphin, B. (1999) *Nat. Biotechnol.* **17,** 1030–1032
38. Shevchenko, A., Wilm, M., Vorm, O., and Mann, M. (1996) *Anal. Chem.* **68,** 850–858
39. Schirle, M., Heurtier, M. A., and Kuster, B. (2003) *Mol. Cell Proteomics* **2,** 1297–1305
40. Perkins, D. N., Pappin, D. J., Creasy, D. M., and Cottrell, J. S. (1999) *Electrophoresis* **20,** 3551–3567
41. Mann, M., and Wilm, M. (1994) *Anal. Chem.* **66,** 4390–4399
42. Sanz, L., Sanchez, P., Lallena, M. J., Diaz-Meco, M. T., and Moscat, J. (1999) *EMBO J.* **18,** 3044–3053
43. Drewes, G., and Nurse, P. (2003) *FEBS Lett.* **554,** 45–49
44. Mollinari, C., Lange, B., and Gonzalez, C. (2002) *Biol. Cell* **94,** 1–13
45. Trinczek, B., Brajenovic, M., Ebneth, A., and Drewes, G. (2004) *J. Biol. Chem.* **279,** 5915–5923
46. Sontag, E., Nunbhakdi-Craig, V., Lee, G., Brandt, R., Kamibayashi, C., Kuret, J., White, C. L., 3rd, Mumby, M. C., and Bloom, G. S. (1999) *J. Biol. Chem.* **274,** 25490–25498
47. Baas, A. F., Boudeau, J., Sapkota, G. P., Smit, L., Medema, R., Morrice, N. A., Alessi, D. R., and Clevers, H. C. (2003) *EMBO J.* **22,** 3062–3072
48. Nozaki, M., Onishi, Y., Togashi, S., and Miyamoto, H. (1996) *DNA Cell Biol.* **15,** 505–509
49. Clark, K. A., McGrail, M., and Beckerle, M. C. (2003) *Development* **130,** 2611–2621
50. Muslin, A. J., Tanner, J. W., Allen, P. M., and Shaw, A. S. (1996) *Cell* **84,** 889–897
51. Boudeau, J., Sapkota, G., and Alessi, D. R. (2003) *FEBS Lett.* **546,** 159–165
52. Drewes, G., and Bouwmeester, T. (2003) *Curr. Opin. Cell Biol.* **15,** 199–205
53. Gavin, A. C., Bosche, M., Krause, R., Grandi, P., Marzioch, M., Bauer, A., Schultz, J., Rick, J. M., Michon, A. M., Cruciat, C. M., Remor, M., Hofert, C., Schelder, M., Brajenovic, M., Ruffner, H., Merino, A., Hudak, M., Dickson, D., Rudi, T., Gnau, V., Bauch, A., Bastuck, S., Huhse, B., Leutwein, C., Heurtier, M. A., Copley, R. R., Rybin, V., Drewes, G., Raida, M., Bouwmeester, T., Bork, P., Seraphin, B., Kuster, B., Neubauer, G., and Superti-Furga, G. (2002) *Nature* **415,** 141–147
54. Gao, L., Macara, I. G., and Joberty, G. (2002) *Gene* (*Amst.*) **294,** 99–107
55. Muller, J., Ory, S., Copeland, T., Piwnica-Worms, H., and Morrison, D. K. (2001) *Mol. Cell* **8,** 983–993
56. Peng, C. Y., Graves, P. R., Ogg, S., Thoma, R. S., Byrnes, M. J., 3rd, Wu, Z., Stephenson, M. T., and Piwnica-Worms, H. (1998) *Cell Growth Differ.* **9,** 197–208
57. Nishigaki, K., Thompson, D., Yugawa, T., Rulli, K., Hanson, C., Cmarik, J., Gutkind, J. S., Teramoto, H., and Ruscetti, S. (2003) *J. Biol. Chem.* **278,** 13520–13530
58. Schneider, A., Laage, R., von Ahsen, O., Fischer, A., Rossner, M., Scheek, S., Grunewald, S., Kuner, R., Weber, D., Kruger, C., Klaussner, B., Gotz, B., Hiemisch, H., Newrzella, D., Martin-Villalba, A., Bach, A., and Schwaninger, M. (2004) *J. Neurochem.*, in press

Molecular Basis of Cell and Developmental Biology:

Comprehensive Proteomic Analysis of Human Par Protein Complexes Reveals an Interconnected Protein Network

Miro Brajenovic, Gerard Joberty, Bernhard Küster, Tewis Bouwmeester and Gerard Drewes

J. Biol. Chem. 2004, 279:12804-12811.
doi: 10.1074/jbc.M312171200 originally published online December 15, 2003