Cellular Redistribution of Protein Tyrosine Phosphatases LAR and PTPσ by Inducible Proteolytic Processing

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Abstract. Most receptor-like protein tyrosine phosphatases (PTPases) display a high degree of homology with cell adhesion molecules in their extracellular domains. We studied the functional significance of processing for the receptor-like PTPases LAR and PTPσ. PTPσ biosynthesis and intracellular processing resembled that of the related PTPase LAR and was expressed on the cell surface as a two-subunit complex. Both LAR and PTPσ underwent further proteolytical processing upon treatment of cells with either calcium ionophore A23187 or phorbol ester TPA. Induction of LAR processing by TPA in 293 cells did require overexpression of PKCα. Induced proteolysis resulted in shedding of the extracellular domains of both PTPases. This was in agreement with the identification of a specific PTPσ cleavage site between amino acids Pro_{821} and Ile_{822}. Confocal microscopy studies identified adherens junctions and desmosomes as the preferential subcellular localization for both PTPases matching that of plakoglobin. Consistent with this observation, we found direct association of plakoglobin and β-catenin with the intracellular domain of LAR in vitro. Taken together, these data suggested an involvement of LAR and PTPσ in the regulation of cell contacts in concert with cell adhesion molecules of the cadherin/catenin family. After processing and shedding of the extracellular domain, the catalytically active intracellular portions of both PTPases were internalized and redistributed away from the sites of cell–cell contact, suggesting a mechanism that regulates the activity and target specificity of these PTPases. Calcium withdrawal, which led to cell contact disruption, also resulted in internalization but was not associated with prior proteolytic cleavage and shedding of the extracellular domain. We conclude that the subcellular localization of LAR and PTPσ is regulated by at least two independent mechanisms, one of which requires the presence of their extracellular domains and one of which involves the presence of intact cell–cell contacts.

A key element in the regulation of cell–cell and cell–matrix contacts is the tyrosine phosphorylation of proteins that are localized in focal adhesions and at intercellular junctions (for reviews see Kemler, 1993; Clark and Brugge, 1995). While much is known about the protein tyrosine kinases involved in the phosphorylation of cell adhesion components, very little information exists about the identity of protein tyrosine phosphatases (PTPases),[1] which are responsible for the dephosphorylation and thereby regulation of these structural complexes. Probable candidates are those receptor-like PTPases that contain cell adhesion molecule-like extracellular domains and could therefore regulate their intrinsic phosphatase activity in response to cell contact. Recent reports suggest that some PTPases do, in fact, possess properties that resemble those of classical cell adhesion molecules (for review see Brady-Kalnay and Tonks, 1995). A direct involvement in cell–cell contact has so far been demonstrated for PTPμ (Brady-Kalnay et al., 1993; Gebbink et al., 1993) and PTPκ (Sap et al., 1994), for which a homophilic interaction between their extracellular domains was found. The localization of PTPμ (Brady-Kalnay et al., 1995; Gebbink et al., 1995), PTPκ (Fuchs et al., 1996), and PCP-2 (Wang et al., 1996) was restricted to sites of cell–cell contact and surface expression of PTPμ (Gebbink et al., 1995), and PTPκ (Fuchs et al., 1996) was increased in a cell density-dependent manner. Moreover, a direct association of PTPκ (Fuchs et al., 1996) and PTPμ (Brady-Kalnay et al., 1995) with members of the cadherin/catenin family suggests that proteins of the cell adhesion complex represent physiological substrates for these PTPases. A possible regulatory function in cell–matrix adhesion has been proposed for LAR, another receptor-like PTPase, which associated with focal cell–substratum adhesions via the newly identified LAR interacting protein 1, LIP-1 (Serra-Pages et al., 1995).

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1. *Abbreviations used in this paper*: GST, glutathione-S-transferase; PTPase, protein tyrosine phophatase.

PTPμ (Gebbink et al., 1991), PTPκ (Jiang et al., 1993; Fuchs et al., 1996), PTPδ (Krueger et al., 1990; Mizuno et al., 1993, Pulido et al., 1995*a*), PCP-2 (Wang et al., 1996), and LAR (Streuli et al., 1988, Pot et al., 1991) are members of the so-called type II receptor-like PTPases. The extracellular domains of these PTPases contain a variable number of Ig-like and fibronectin type III-like (FNIII) domains (for review see Charbonneau and Tonks, 1992). With the exception of PCP-2 (Wang et al., 1996), these PTPases also share characteristics in their biosynthesis. They all underwent proteolytic processing by a furin-like endoprotease and were expressed at the cell surface in two subunits which were not covalently linked (Streuli et al., 1992; Yu et al., 1992; Jiang et al., 1993; Brady-Kalnay and Tonks, 1994; Gebbink et al., 1995; Pulido et al., 1995*a*; Fuchs et al., 1996). It was shown for LAR that the E subunit, which contains the cell adhesion molecule-like extracellular domain, was shed from the cell surface when cells were grown to a high density (Streuli et al., 1992). This shedding of the E subunit of LAR was the result of an additional proteolytic processing step that could also be induced by treatment of the cells with the phorbol ester TPA (Serra-Pages et al., 1995). An accumulation of E subunits in the supernatant of cells was also observed for PTPμ (Gebbink et al., 1995) and PTPδ (Pulido et al., 1995*a*), and this suggests a common mechanism in the regulation of type II PTPases. However, the effect of proteolytic processing on either the catalytic activity, the substrate specificity, or the cellular localization of these PTPases has not yet been determined.

We report here that PTPσ, a recently identified new member of the family of receptor-like type II PTPases (Pan et al., 1993; Walton et al., 1993; Yan et al., 1993; Ogata et al., 1994; Zhang et al., 1994), underwent biosynthesis and proteolytic processing in a manner that resembled that of the most closely related PTPase LAR. Moreover, further proteolytic processing of PTPσ as well as of LAR could be induced by treatment of the cells with TPA or the calcium ionophore A23187. Transient expression studies indicated that TPA-induced processing of LAR, but not PTPσ, was dependent on the coexpression of PKCα. Inducible processing of both PTPases took place in the extracellular segment of the P subunit in a juxtamembrane position and led to the shedding of the E subunit. Both LAR and PTPσ were predominantly localized in regions of cell–cell contact and accumulated in dot-like structures that could be identified as adherens junctions and desmosomes by colocalization with plakoglobin (Cowin et al., 1986). Moreover, plakoglobin and β-catenin, another component of E-cadherin–containing cell adhesion complexes in adherens junctions, associated directly with the intracellular domain of LAR in vitro. The inducible shedding of the E subunit of LAR and PTPσ was followed by a redistribution of the PTPases within the cell membrane and by an internalization of the cleaved P subunits. It therefore represents a mechanism through which the phosphatase activity of these PTPases could be regulated in response to cell–cell contact. The cell adhesion molecule-like character of LAR and PTPσ was further supported by the fact that the internalization of LAR and PTPσ occurred independently of the proteolytic processing if cells were grown in calcium-depleted growth medium. The analogies in specific localization as well as internalization behavior of PTPσ and LAR, with molecules of the cadherin/catenin family, strongly suggest a direct involvement of PTPσ and LAR in the formation or maintenance of intercellular contacts.

Materials and Methods

Cell Lines and Culture Media

A431 (CRL 1555; American Type Culture Collection, Rockville, MD) and HeLa (CCL 2; American type Culture Collection) cells were grown in Dulbeco's minimal essential medium (DMEM) containing 4.5 mg/ml glucose and supplemented with 10% FCS. For growth of 293 cells (CRL 1573; American Type Culture Collection), DMEM containing 1.0 mg/ml glucose and 10% FCS was used. All growth media were supplemented with 2 mM L-glutamine before use. For starvation experiments, A431 and HeLa cells were grown for 48 h and 293 cells for 24 h in their respective growth media, which were diluted 1:40 with identical serum-free medium. All media and supplements were purchased from GIBCO BRL (Eggenstein, Germany).

cDNA Constructs

For transient expression experiments, the human LAR cDNA was cloned into the cytomegalovirus early promoter-based (Eaton et al., 1986) expression plasmid pRK5. For subcloning purposes, pSP65–LAR was kindly provided by H. Saito (Harvard Medical School, Boston, MA). pSP65–LAR was cut with restriction enzymes EcoRI and NruI, and the two resulting fragments of 4,448 (EcoRI/EcoRI) and 2,004 bp (EcoRI/NruI) containing the complete coding region of human LAR were inserted in the pRK5 plasmid, which had been linearized with restriction enzymes EcoRI and EcoRV. The pRK5 expression plasmid containing the cDNA of rat PTPσ (Yan et al., 1993) was kindly provided by Y. Schlessinger (New York University Medical Center, New York).

The plasmid coding for the GST–hPTP LAR_i fusion protein was constructed by amplification of the cDNA sequence between amino acids 1,259 to 1,881 of human LAR using PCR with oligonucleotides 5′-CATGGATCCAAAAAGGAAAAGGACCCAC-3′ and 5′-GATCAGATCTTCACGTTGCATAGTGGTCAAAGC-3′. The PCR product was cut with restriction enzymes BamHI and BglII and was inserted in the appropriate pGEX vector (Pharmacia Biotech, Uppsala, Sweden). Human β-catenin and plakogobin (these sequence data are available from GenBank/EMBL/DDBJ under accession number Z19054 and M23410, respectively) were amplified from cDNA generated from MCF7 cells using the PCR method and were cloned in pRK5 expression plasmid. The integrity of subcloned PCR products was confirmed by sequence analysis. The CMV-driven expression plasmid for PKCα and rabbit antiserum 105 directed against PKCα were described elsewhere (Seedorf et al., 1995).

Antibodies

Rabbit antisera α LAR_{EN} and α LAR_{EC} were generated against synthetic peptides corresponding to NH_2 (amino acids 5–18) and COOH-terminal (amino acids 1,129–1,142) regions of the LAR E subunit, respectively. Rabbit antisera 320 and 322 were kindly provided by Y. Schlessinger (New York University Medical Center, New York). Antiserum 320 is directed against a peptide corresponding to the COOH-terminus of LAR (amino acids 1868–1881) and PTPσ (amino acids 1465–1478), whereas antiserum 322 is directed against a peptide corresponding to the NH_2 terminus of PTPσ (amino acids 5–18). Anti-plakoglobin (γ-catenin) and anti-β-catenin antibodies were purchased from Transduction Laboratories (Lexington, KY).

Transient Expression in 293 Cells and Stimulation of Cells

293 cells were seeded in 20% confluency and were transfected 24 h later using the calcium phosphate precipitation technique described by Chen and Okayama (1987). 16 h after transfection, cells were washed once with starvation medium (DMEM with 0.25% FCS) and grown for an additional 24 h in the same medium. Alternatively for metabolic labeling with [^{35}S]methionine, cells were washed and grown in methionine-free minimal

essential medium with 0.25% dialyzed FCS. 50 μCi/ml [^{35}S]methionine (1,000 Ci/mmol, Amersham Intl., Amersham, UK) were added 16 h before lysis. Before lysis, cells were stimulated with 10^{-5} M calcium ionophore A23187 (Sigma Chemical Co., Taufkirchen, Germany), 1 μM phorbol ester TPA (Sigma Chemical Co.), 5 mM EGTA, or 30 μM calpeptin (Calbiochem, Bad Soden, Germany). Pervanadate was freshly prepared from sodiumorthovanadate and H_2O_2 and was used in a final concentration of 0.1 mM Na_3V0_4 and 3×10^{-7} M H_2O_2. Time intervals of incubation are given in the figure legends.

Immunoprecipitation and Immunoblotting

Cells were washed once with ice-cold PBS and lysed in Triton X-100 lysis buffer (50 mM Hepes, pH 7.2, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 100 mM NaF, 10 mM $Na_4P_2O_7$, 2 mM Na_3V0_4, 5 mM EGTA, 1 mM PMSF, 1 μg/ml each leupeptin, pepstatin, antipain, and chymostatin). Lysates were centrifuged for 20 min at 12,500 *g* to obtain the supernatant fraction, and protein concentration was determined by using the method described by Bradford (1976). Equal amounts of proteins were used in each experiment. For immunoprecipitation, protein A-Sepharose (Pharmacia Biotech) was preincubated with specific antisera, washed twice with HNTG (50 mM Hepes, pH 7.2, 150 mM NaCl, 10% glycerol, 0.1% Triton X-100, 1 mM Na_3V0_4, 1 mM PMSF), and added to the lysates. For binding to WGA-Sepharose (Sigma Chemical Co.), lysates were diluted 1:5 in HNTG and tissue culture supernatants centrifuged twice at 1,000 *g* for 15 min before adding WGA-Sepharose. Glutathione-S-transferase (GST) fusion proteins were expressed in *Escherichia coli* and purified as described (Smith and Johnson, 1988). 3 μg of GST–hPTP LAR_i fusion protein and a threefold molar excess of GST were incubated with equal amounts of cell lysates and immobilized by adding glutathione–Sepharose (Sigma Chemical Co.). All immobilization steps were performed for 4–16 h, and the resulting complexes were washed three times with HNTG. Samples were boiled in SDS sample buffer for 10 min followed by separation in SDS-PAGE. For immunoblotting analysis, the enhanced chemiluminescence system (Amersham Intl.) was used in conjunction with goat anti–rabbit antibodies (Bio Rad Labs). For reprobing purposes, blots were stripped in 62.5 mM Tris/HCl, pH 6.8, 2% SDS, and 100 mM β-mercaptoethanol at 50°C for 1 h.

NH_2-terminal Sequencing

PTPσ was expressed transiently in 293 cells, and the cells were incubated for 1 h with 10^{-5} M A23187 before lysis in Triton X-100 lysis buffer described above, without the phosphatase inhibitors NaF, $Na_4P_2O_7$, and Na_3V0_4. Lysates of four 15-cm tissue culture plates were immunoprecipitated with antiserum 320, immunprecipitates separated in 8% SDS-PAGE, and transferred to ProBlot™-membrane (Applied Biosystems). Proteins were stained with Coomassie blue R-250, and the processing product of the PTPσ P subunit was isolated. Microsequencing was performed by using a sequencer (model 494; Applied Biosystems) using standard reagents and programs as suggested by the manufacturer.

Immunofluorescence Microscopy

For immunofluorescence studies, A431 cells (CRL 1555; American Type Culture Collection) were grown for 48 h on uncoated glass coverslips to different degrees of confluency. Control cells or cells incubated with either TPA, EGTA, or ionophore for the respective time intervals (see Figs. 7–10, legends) were fixed with 2% formaldehyde freshly prepared from paraformaldehyde in PBS (pH 7.4, 0.12 M sucrose). Autofluorescence was quenched with PBS glycine (100 mM), and the cells were permeabilized with 0.5% saponin in PBS (5 min). Unspecific antibody binding was blocked for 1 h with phosphate buffered gelatine (PBG: PBS, 0.5% bovine serum albumin, 0.045% cold-water fish gelatine). Primary antibody incubation was done at room temperature for 2 h after dilution in PBG, 1:50 for rabbit antisera α LAR_{EN}, 320, and 322, and 1:200 for monoclonal anti-plakoglobin antibody. After three washes in PBG, primary antibody binding was detected with isotype-specific secondary antibody, FITC(DTAF)-conjugated donkey-anti–rabbit IgG (1:200), or Cy3-conjugated goat-anti–mouse IgG (1:300; Jackson ImmunoResearch Laboratories, West Grove, PA). For double labeling experiments, antibody decoration was done consecutively. Controls were incubated with either species-specific nonimmune serum or with secondary antibody alone. Coverslips were mounted under glycerol–2.4% Dabco (1,4 Diazabicyclo [2.2.2*octane]) and were viewed with appropriate band pass filters on a laser confocal microscope (LSM 410; Carl Zeiss, Oberkochen, Germany) using a 40× oil immersion objective of aperture 1.3. Images were recorded with a voxel size of 0.082 mm and smoothed for printouts by subdividing the pixels and linear interpolation. Controls were recorded at identical settings. To visualize the localization of antibody binding together with the cellular morphology, a gray scale transmission image (pseudo-phase contrast) and the two confocal fluoresence images (FITC and Cy3) were superimposed in AVS (Advances Visual Systems, Waltham, MA).

Scanning Electron Microscopy

For scanning electron microscopy, cells were fixed with 2% formaldehyde/1% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4, 0.12 M sucrose on ice), postfixed with 0.02 M osmium tetroxide for 30 min, flushed with 30% ethanol, and dehydrated through graded ethanol and 100% dried acetone. After critical point drying with CO_2 for 60 min (Polaron, Watford, UK) the coverslips were attached to scanning electron microscopy stubs with conductive carbon paint and left overnight. Before examination on a scanning electron microscope (35; Jeol, Tokyo, Japan) they were gold sputtered to a thickness of 20 nm (SCD 020 Coating Unit; Balzers, Liechtenstein).

Results

Biosynthesis and Processing of LAR and PTPσ

LAR and PTPσ are highly related PTPases whose rat homologues display a sequence identity of 79% in their proximal membrane PTPase domain, 90% in their COOH-terminal PTPase domain, and 57% in their extracellular domain (Zhang et al., 1994). LAR contains three Ig- and eight FNIII-like domains in the extracellular domain and two intracellular PTPase domains. Three splice variants of PTPσ are known so far. The rat protein we analyzed (Pan et al., 1993; Walton et al., 1993; Yan et al., 1993) differs from LAR in so far as it lacks the FNIII-like domains four through seven. While it was shown that LAR was expressed in two subunits (Streuli et al., 1992; Yu et al., 1992), the biosynthesis of PTPσ has not yet been studied. We hypothesized that PTPσ would be processed in a manner that is analogous or similar to the processing of LAR because a polyclonal antiserum directed against one of its FNIII-like domains recognized a protein of ~100 kD instead of the 168 kD that would have been predicted from the full length sequence of PTPσ (Yan et al., 1993; Rotin et al., 1994). Fig. 1 *A* shows the schematic structure of LAR and PTPσ, the proposed biosynthesis of PTPσ, and the recognition sites of subunit-specific antibodies used in this study.

To compare the biosynthesis of human LAR and rat PTPσ, both proteins were transiently expressed in human embryonic kidney 293 cells (Fig. 1, *B* and *C*). As shown in Fig. 1 *B*, antiserum (320) directed against the identical COOH terminus of human LAR and rat PTPσ recognized proteins of 205 and 84 kD in cell lysates of LAR expressing 293 cells and proteins of 158 and 80 kD in PTPσ-expressing 293 cells. These protein bands represented the precursor and the proteolytically processed P subunits of both PTPases, respectively. The reduced size of the PTPσ precursor and E subunit of 40 kD was in good agreement with the presence of only four FNIII-like domains instead of eight such domains in LAR. The molecular mass of the P subunit of PTPσ, on the other hand, varied only by 4 kD. The large amount of unprocessed precursor protein that was detected is most likely the result of overexpression in the 293 cell system.

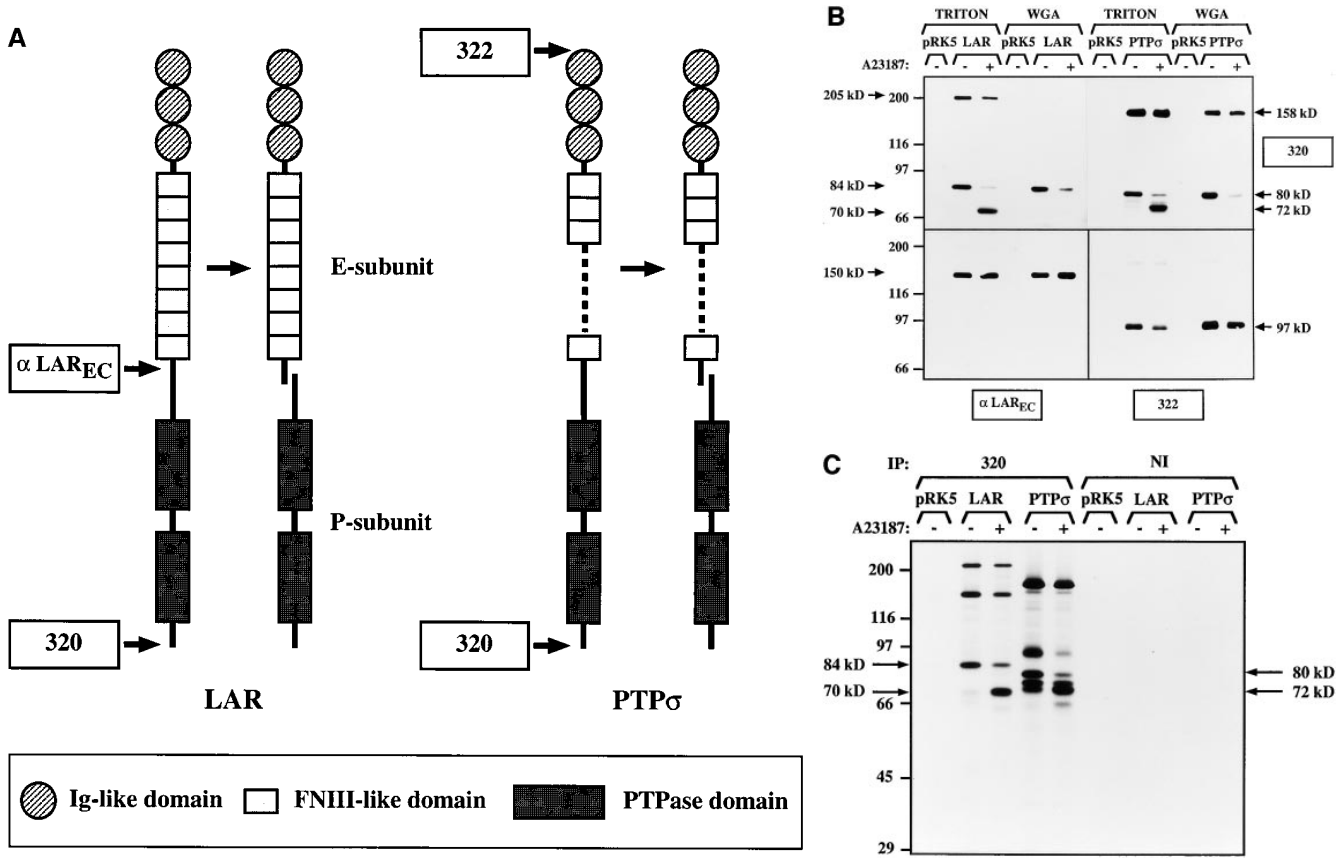

Figure 1. Physiological- and calcium ionophore-induced processing of LAR and PTPσ after transfection into 293 cells. (*A*) Schematic representation of the biosynthesis of LAR and PTPσ. Dashed lines indicate a gap introduced for alignment purposes. Antibody 320 specifically recognizes the P subunit, while α LAR_{EC} and 322 recognize the E subunits of LAR and PTPσ, respectively. (*B*) LAR or PTPσ were transiently expressed in 293 cells and before lysis cells were treated for 1 h with or without 10^{-5} M A23187, as indicated. Control cells were transfected with the expression plasmid pRK5. Lysates were either separated by 8% SDS-PAGE (*TRITON*) or after binding to WGA–sepharose beads. Proteins were transferred to nitrocellulose and analyzed by immunoblotting of the membrane with antisera specific for the COOH terminus of LAR and PTPσ (*320*), the NH_2 terminus of PTPσ (*322*), or the COOH terminus of the LAR E subunit (αLAR_{EC}). Arrows on the left indicate molecular weight standards, and arrows on the right indicate the position of the LAR and PTPσ subunits. (*C*) 293 cells transfected with LAR, PTPσ, or control plasmid were labeled with [^{35}S]methionine (16 h) and incubated with or without A23187, as described above. Lysates were immunoprecipitated with antiserum specific for LAR and PTPσ (*320*) or with nonimmune serum (*NI*). Immunoprecipitates were separated by 8% SDS-PAGE, and the dried gel was exposed to X-ray film for 24 h. Arrows on the left indicate molecular weight and on the right the position of the P subunits of LAR and PTPσ, respectively.

The proteins immunoprecipitated with the same antiserum from [^{35}S]methionine-labeled cells were identical (Fig. 1 *C*). As previously demonstrated with LAR (Streuli et al., 1992; Yu et al., 1992), the noncovalent linkage between the E and P subunit was stable under standard cell lysis conditions and during immunoprecipitation. The LAR E subunit of 150 kD could thus be coimmunoprecipitated with the antiserum directed against the P subunit (Fig. 1 *C*). For PTPσ, a coimmunoprecipitated 97-kD protein (Fig. 1 *C*) was identified as its processed extracellular domain by immunoblot analysis with an antiserum raised against an NH_2-terminal peptide (Fig. 1 *B*). Taken together, these data show that the biosynthesis of PTPσ is indeed comparable to that of LAR in every aspect (Fig. 1 *A*).

When 293 cells that overexpressed the PTPases were treated with the calcium ionophore A23187 before lysis, additional proteins of 70 (LAR) and 72 kD (PTPσ) could be immunoprecipitated with the COOH terminus-specific consensus antiserum 320. In addition, the amount of immunoprecipitated P and E subunits was considerably reduced, whereas the amount of immunoprecipitated precursor of both PTPases was not or much less affected (Fig. 1 *C*). Immunoblot studies using specific antisera directed against the E and P subunit indicated that the 70- and 72-kD proteins were derived from the P subunits of LAR and PTPσ, respectively, by proteolytic processing at the NH_2 terminus (Fig. 1 *B*). Since coimmunoprecipitation of the E subunits with the shortened P subunits was not detected (Fig. 1 *C*), proteolytic processing induced by calcium ionophore treatment resulted in separation of the E and the P subunit. This lack of association between the E subunit and the shortened P subunit could also be demonstrated by analyzing the binding of subunits of LAR and PTPσ to WGA (Fig. 1 *B*). In untreated cells, the P subunits of LAR and PTPσ were found enriched in the fraction of WGA-bound proteins. However, after processing was induced, the 70- and 72-kD protein bands were no longer detected in the WGA-bound protein fraction (Fig. 1 *B*). This indi-

cates that the P subunits would have to be linked to their respective E subunits to be detected in the WGA-bound protein fraction.

The antiserum specific for the COOH terminus of the PTPases recognized an additional protein of 76 kD in 293 cells that expressed PTPσ. The relative amount of this protein varied from experiment to experiment and was not affected by calcium ionophore treatment of the cells (Fig. 1, *B* and *C*). It therefore most likely represents a degradation product of PTPσ, although a different type of processing cannot be excluded. An equivalent protein product, however, could not be detected in 293 cells that expressed LAR.

Involvement of PKCα in the Proteolytic Processing of LAR and PTPσ

Proteolytic processing of transmembrane proteins could be shown to depend on the activation of PKCα in several instances (for review see Ehlers and Riordan, 1991). We therefore investigated whether treatment of LAR and PTPσ overexpressing 293 cells with the PKCα activator TPA (12-*O*-tetradecanoylphorbol–13-acetate) could induce proteolytic processing of these PTPases. In addition we analysed if this processing is dependent on the coexpression of PKCα in these cells. 293 cells were transfected with vectors encoding LAR or PTPσ alone or together with a PKCα expression plasmid. The effect of TPA treatment of these cells on the proteolytic processing of the PTPases was determined by immunoblotting of cell lysates with the COOH terminus-specific antiserum 320 (Fig. 2, *top gel*). The reprobing of the same membrane with an antiserum specific for PKCα confirmed comparable expression levels of PKCα (Fig. 2, *bottom gel*). For immunoblotting we used smaller quantities of cell lysate from cells that expressed only the PTPases, because coexpression of PKCα consistently reduced the amount of LAR and PTPσ expression in these cells.

As shown in Fig. 2, induced processing of overexpressed LAR in 293 cells occurred only when PKCα was coexpressed. This indicated a critical involvement of this enzyme in the TPA-induced processing of LAR. PTPσ processing in response to TPA, on the other hand, was independent of PKCα overexpression in 293 cells. We cannot exclude that this effect was mediated by endogenous PKCα and may be due to a higher susceptibility of PTPσ towards TPA-induced processing in comparison to LAR.

In TPA-treated cells the P subunits of LAR, whether processed or unprocessed, showed a shift to a higher molecular weight in comparison to nontreated cells or to cells in which processing was induced by calcium ionophore (Fig. 2). In contrast to the TPA-induced proteolytic processing of LAR, this shift to a higher molecular weight of the noncleaved P subunit occured even in the absence of overexpressed PKCα and was most likely mediated by endogenous PKCα. We assume that the increase in molecular weight was due to a modification of the LAR P subunits by serine/threonine phosphorylation because we observed TPA-dependent [^{32}P]orthophosphate incorporation in PKCα coexpressing 293 cells (data not shown). A shift to higher molecular weight for the P subunits of PTPσ, however, was not observed (Fig. 2) although TPA-induced [^{32}P]orthophosphate incorporation in the P subunits of PTPσ was comparable to LAR (data not shown).

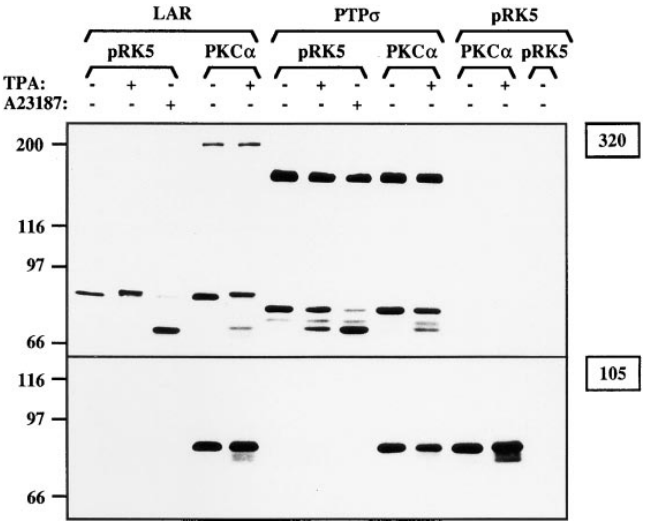

Figure 2. Effect of TPA treatment and coexpression of PKCα on the processing of LAR and PTPσ in 293 cells. LAR or PTPσ were transiently expressed in 293 cells either alone or together with PKCα. As controls, pRK5 plasmid or PKCα expressing cells were used. Before lysis, cells were treated for 1 h with or without 1 μM TPA or 10^{-5} M A23187 as indicated. Lysates were separated by 7% SDS-PAGE (TRITON), proteins were transferred to nitrocellulose and subsequently analyzed by immunoblotting of the same membrane with antisera specific for the COOH terminus of LAR and PTPσ (*320*) and PKCα (*105*). The amount of cell lysates analyzed is one third in LAR/pRK5-transfected cells and one half in PTPσ/pRK5 transfected cells in comparison to lysates in the other lanes. Sizes of molecular weight standards in kD are indicated on the left.

Processing of LAR and PTPσ in HeLa, A431, and 293 Cells

In human platelets, cytosolic PTP1B was proteolytically processed after treatment with A23187 and TPA. This processing occurred in a calcium-dependent manner and was mediated by the intracellular protease calpain (Frangioni et al., 1993). Therefore, we studied the calcium dependence as well as the influence of a specific calpain inhibitor, calpeptin (Tsujinaka et al., 1988), on the proteolytic processing of LAR and PTPσ (Fig. 3). Accordingly, A431, HeLa cells, and nontransfected 293 cells were treated with the calcium ionophore A23187 or with TPA. Cells that were treated with A23187 were preincubated either with calpeptin or with an excess of EGTA to deplete the medium of calcium. Lysates of the cells were immunoprecipitated with antiserum 320 and immunoprecipitates analyzed in immunoblots with antiserum 320 and antiserum αLAR_{EC} (Fig. 3).

In the cell lines, we examined the expression of PTPσ with E subunit-specific (data not shown in Fig. 3; see Fig. 5) as well as P subunit-specific antisera could be clearly detected only in A431 cells, whereas all three cell lines express LAR (Fig. 3). Ionophore-induced proteolytic processing led in all cell lines to protein products of the same molecular weight. Pretreatment with EGTA eliminated the appearance of the proteolytic products, suggesting that proteolysis was indeed calcium dependent. However, pretreatment with the calpain inhibitor calpeptin had no effect, thereby indicating that calpain was not required for

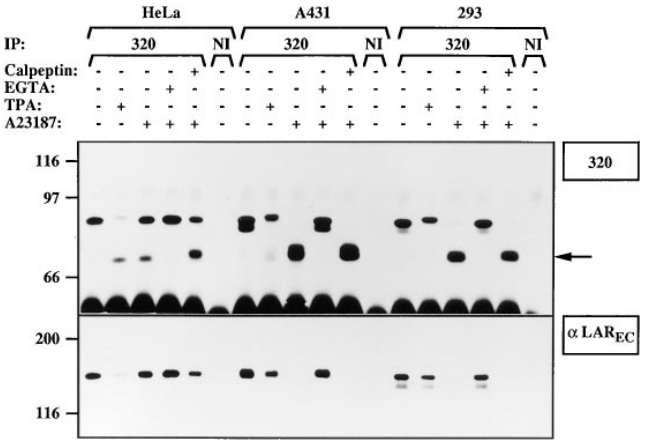

Figure 3. Characteristics of inducible processing of LAR and PTPσ in different cell lines. HeLa, A431, and 293 were incubated with or without A23187 (10^{-5} M, 1 h) or TPA (1 μM, 1 h) or were pretreated with EGTA (5 mM, 1h) or calpeptin (30 μM, 1 h). Cell lysates (1.2 mg protein) were immunoprecipitated with either antiserum specific for LAR and PTPσ (320) or nonimmune serum (*NI*). Immunoprecipitates were separated by 8% SDS-PAGE, transferred to nitrocellulose, and analyzed by immunoblotting of the membrane with antiserum specific for the COOH terminus of LAR and PTPσ (*320*) or the COOH terminus of the LAR E subunit (αLAR_{EC}).

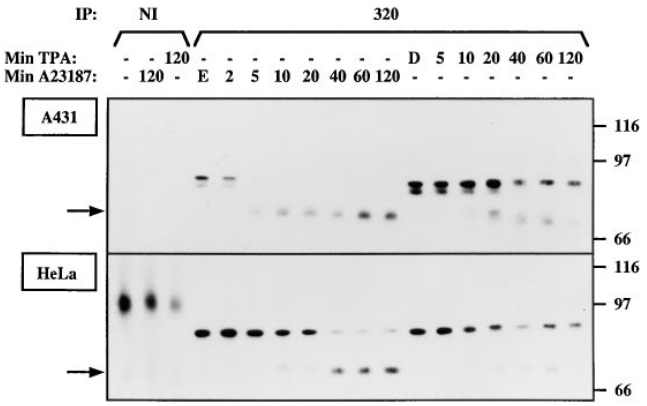

Figure 4. Time course of inducible processing of LAR and PTPσ in HeLa and A431 cells. A431 and HeLa cells were starved for 2 d and then incubated for different time intervals (indicated in min) with A23187 (10^{-5} M) or TPA (1 μM). Control cells were incubated with vehicle, which is indicated as E (ethanol) and D (DMSO). Cell lysates (0.9 mg protein) were immunoprecipitated with antiserum specific for LAR and PTPσ (*320*) or nonimmune serum (*NI*). Immunoprecipitates were separated by 8% SDS-PAGE, transferred to nitrocellulose, and analyzed by immunoblotting with antiserum specific for the COOH terminus of LAR and PTPσ (*320*). Arrows at the left indicate the position of the processing products of the P subunit of LAR and PTPσ.

this proteolytic effect. A control experiment using PTP1B transiently expressed in 293 cells showed that the same concentration of calpeptin could inhibit PTP1B processing almost completely (data not shown). This confirmed that, although the ionophore-induced processing of LAR and PTPσ was calcium dependent, it was not mediated by calpain.

In agreement with the results obtained with 293 cells (Fig. 2), the treatment of cells with TPA resulted in proteolytic processing and differed from the one induced by ionophore treatment, since it caused a shift of the P subunits as well as the processing products to a higher molecular weight. As opposed to overexpressed LAR in 293 cells (Fig. 2), the inducible processing of endogenous LAR by TPA treatment in the same cells as well as in HeLa and A431 cells occurred without transfection of PKCα and is therefore most likely mediated by the endogenous enzyme in these cells. As seen with the ionophore, TPA-induced processing occurred at the NH_2 terminus of the P subunits and cleaved the linkage between the E and the P subunits (Fig. 3). Moreover, treatment with A23187 and TPA induced an identical proteolytic processing in SK-BR-3, BT-20, MIA-PaCa-2, and PC12 cells (data not shown). This suggested that this inducible proteolytic processing pattern is a general feature of LAR and PTPσ and occurs independently of cell type.

Time Course of LAR and PTPσ Processing in A431 and HeLa Cells

We performed a time course study to compare calcium ionophore and TPA-induced processing of LAR and PTPσ in different cell lines. HeLa and A431 cells were incubated for different time periods with A23187 and TPA before lysis, and immunoprecipitates with antiserum 320 were analyzed by immunoblotting with the same antiserum. As shown in Fig. 4, in A431 cells the processing was complete after 10–20 min of ionophore treatment and even on the longest exposures of the immunoblot, the P subunits of the PTPases could not be detected (data not shown). In contrast, in HeLa cells, the processing of LAR was maximal but incomplete after 40 min of treatment and could not be increased further by longer incubation times.

The TPA-induced processing was comparable in both cell lines. Proteolytic processing of both PTPases was maximal after ~40 min and could not be increased further. In A431 cells especially, the lower molecular weight P subunit was affected by TPA treatment. Since the data shown in Fig. 2 already indicated a greater susceptibility of PTPσ to TPA-induced processing, the higher and lower molecular weight bands in A431 cells most likely represented the LAR and PTPσ P subunits, respectively.

It is noteworthy that TPA as well as ionophore treatment for up to 2 h caused the appearance of the same processing products observed after short time treatment. The absence of any additional degradation products after long-term incubation supported the conclusion that proteolytic processing of LAR and PTPσ was a specific and therefore functionally significant event.

Inducible Shedding of LAR and PTPσ-E subunits in A431 Cells

For further characterization, we determined the position of the site where the inducible proteolytic processing takes place. The calculated molecular weight of the intracellular domains and the transmembrane regions of both PTPases was 74 kD. Because the molecular weight of the processing products of the P subunits was 72 and 70 kD (Fig. 1), cleavage most likely occurred within or near the transmembrane region. To determine whether cleavage occurred in the extra- or intracellular part of the P subunits we performed the following experiments. A431 cells were

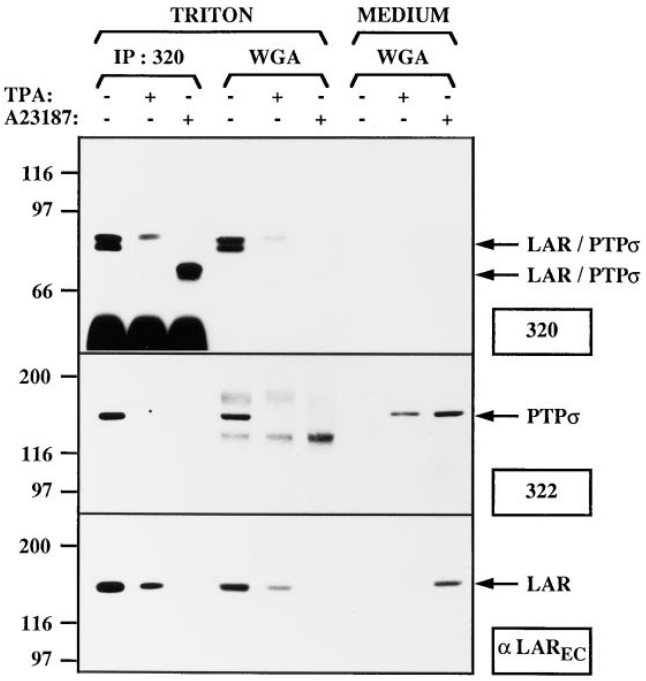

Figure 5. Shedding of LAR and PTPσ E subunits in A431 cells. A431 cells were starved for 2 d, washed once with starvation medium, and treated with or without A23187 (10^{-5} M, 1 h) or TPA (1 μM, 40 min). Cell lysates (*TRITON*) were either immunoprecipitated with antiserum specific for LAR and PTPσ (IP:320, 0.9 mg protein) or were bound to WGA-sepharose beads (WGA, 0.6 mg protein). Proteins of the tissue culture supernatant of these cells (*MEDIUM*, corresponding to 1.5 mg protein of cell lysate) were bound to WGA-sepharose beads (*WGA*). Immunoprecipitates and WGA-bound proteins were separated by 8% SDS-PAGE, transferred to nitrocellulose, and analyzed by immunoblotting of the membrane with antiserum specific for the COOH terminus of LAR and PTPσ (*320*), the NH_2 terminus of PTPσ (*322*), or the COOH terminus of the LAR E subunit (αLAR_{EC}). Arrows at the right indicate the position of the LAR or PTPσ subunits.

treated with vehicle, A23187, or TPA before lysis. Cell lysates were immunoprecipitated with antiserum 320, and glycosylated proteins were bound to WGA–Sepharose. In parallel, glycosylated proteins from the supernatants of the same cells were enriched by binding to WGA–Sepharose. The presence of LAR and PTPσ subunits in the different fractions was analyzed by subsequent immunoblotting with antibodies directed against the different subunits (Fig. 5). As shown in Fig. 5, the E subunits of LAR and PTPσ were no longer present in the fraction of WGA-bound proteins after induced proteolytic processing. Instead, they could be detected in the supernatant. This demonstrated that the cleavage site for induced proteolytic processing was located in the extracellular domain of the P subunit and that processing caused shedding of the E subunits of LAR and PTPσ. As shown earlier, the lower molecular weight band that was recognized by the antibody directed against the COOH terminus of the PTPases was found to be more sensitive to TPA-induced processing (Fig. 4). Because the corresponding E subunit found in the supernatant was recognized by an antiserum specific for PTPσ, the lower molecular weight subunit was now identified as the P subunit of PTPσ.

Shedding of the E subunits of the PTPases could not be observed in overexpressing 293 cells, even though cleavage of the P subunits resulted in loss of the linkage between E and P subunits (Fig. 1, *B* and *C*) comparable to other cell lines (Fig. 3 and 5). This fact is most likely due to inhibition of solubilization of the E subunits by the calcium precipitation technique used for transfection of the cells.

Proteolytic Cleavage Site of PTPσ

Because the inducible processing of LAR and PTPσ was identical in cell lines that endogenously express the phosphatases and in transfected 293 cells, we used the 293 cell overexpression system to isolate the processed P subunit of PTPσ and to determine the cleavage site by NH_2-terminal sequence analysis. The NH_2-terminal sequence (XVDGEEGLI) of the ionophore-induced processing product of PTPσ was identical to amino acids 822-830 of PTPσ, with exception of the first amino acid (X) which could not be identified (Fig. 6). The cleavage occurred extracellulary between amino acids Pro_{821} and Ile_{822}, only six amino acids away from the transmembrane region. In this region the protein sequence of LAR does not show a significant homology to the sequence of PTPσ, and the proteolytic cleavage site of LAR is therefore not necessarily in an analogous position to that of PTPσ. The data in Fig. 5, however, indicated that the inducible processing of LAR also took place in the extracellular part of the P subunit.

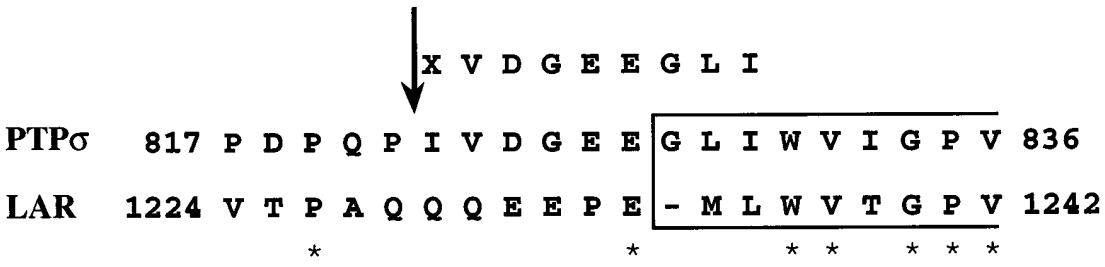

Figure 6. Schematic representation of the calcium ionophore-induced cleavage site of PTPσ in comparison to the corresponding LAR sequence. Shown are the amino acid sequences of PTPσ (amino acids 817–836) and LAR (amino acids 1224–1242). The putative transmembrane regions are boxed, and identical amino acids of both PTPases are indicated by asterisks. The determined NH_2-terminal sequence of the PTPσ processing product is aligned to the homologous PTPσ sequence, and the cleavage site is shown by an arrow.

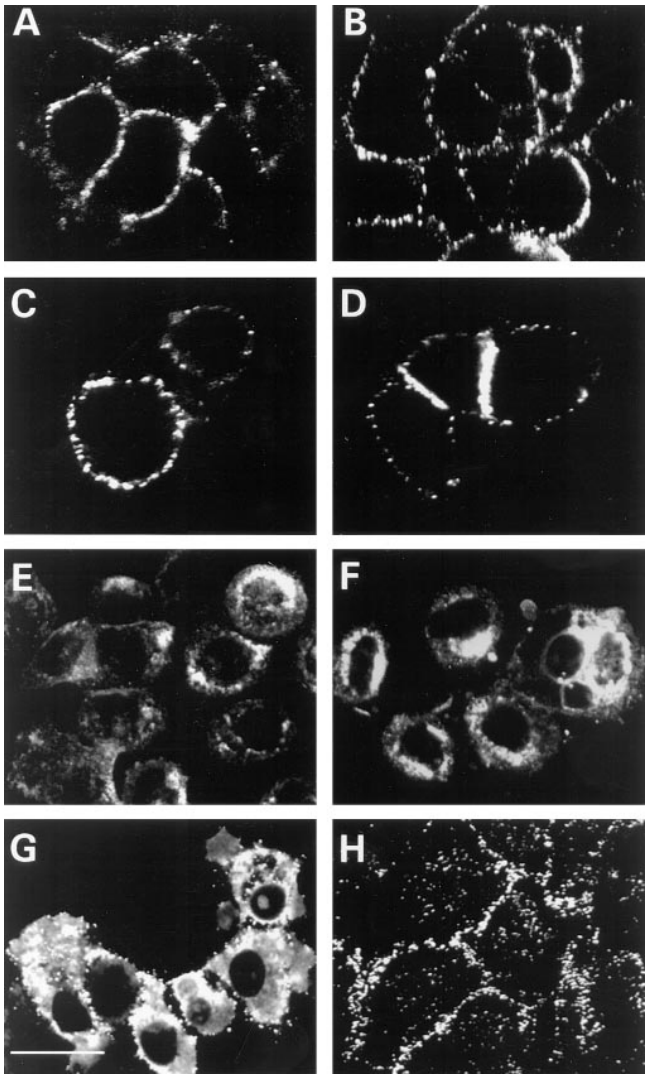

Figure 7. Localization of LAR and PTPσ. A431 cells were grown on glass coverslips, fixed, and labeled for laser confocal microscopy, as indicated in Materials and Methods. *A–D* are representative of unstimulated control cells. *E–H* were treated as follows: (*E*) 5 mM EGTA for 45 min; (*F*) EGTA (5 mM) for 45 min plus A23187 (10^{-5} M) for the last 30 min; (*G*) A23187 (10^{-5} M) for 30 min; (*H*) TPA 1 μM for 40 min. Antibodies were (*A*) α LAR_{EN} (NH_2 terminus of LAR), (*B*) 322 (NH_2 terminus of PTPσ), (*C–H*) 320 (COOH terminus of LAR and PTPσ). Note the concentration of the label along the cell–cell contact sites in *A*, *B*, and *D* and the punctate staining along the circumference of solitary cells (*C*). EGTA induced a ring-shaped core of internalized fluorescent label (*E* and *F*). Ionophore treatment (*G*) resulted in an even cytosolic distribution of antibody labeling. After TPA treatment, some dot-like fluorescence was detected in the cytosol, but predominant labeling remained at intercellular junctions. Bar, 25 μm.

Intracellular Localization of LAR and PTPσ

To study whether the two transmembrane PTPases are associated with any specific subcellular site, we analyzed their localization in A431 cells by immunolabeling and fluorescence laser confocal microscopy. LAR- and PTPσ-specific labeling with either antibodies directed against the intracellular or the extracellular domains of both PTPases was observed in dot-like structures at the attachment sites of the cells to the glass surface (data not shown). Consistent with previous findings for LAR (Serra-Pages et al., 1995), these structures are likely to represent adhesion plaques. In addition, a punctate label along the contact sites of neighboring cells was detected by labeling either the extracellular domain of LAR (Fig. 7 *A*) and PTPσ (Fig. 7 *B*) or the intracellular domain of both PTPases (Fig. 7, *C* and *D*). When cells were grown at low density before cell–cell contacts were formed, labeling was found as punctate staining along the cell membrane (Fig. 7 *C*). Focal concentration of fluorescence was detected at intercellular junctions as soon as these had formed between cells at higher density (Fig. 7 *D*).

Treatment of the cells with EGTA alone (Fig. 7 *E*) or subsequent incubation with ionophore (Fig. 7 *F*), which did not lead to inducible processing of the PTPases (Fig. 3), induced internalization of the intracellular domains of the PTPases. Here fluorescent label was detected as a ring-shaped structure in the perinuclear cytosol, whereas prefixation treatment with ionophore resulted in an internalization of the fluorescence and an even cytosolic staining of the intracellular domains of the PTPases (Fig. 7 *G*). Significant labeling with antibodies directed against the extracellular domains of the PTPases could no longer be detected in ionophore-treated cells (data not shown). Incubation with TPA was associated with only a partial internalization of the P subunits, while a significant fluorescence remained localized in the plasma membrane (Fig. 7 *H*). Concentration of this label at sites of cell–cell contact is reduced in comparison to nontreated cells (Fig. 7 *D*).

The extent of proteolytic processing after treatment of A431 cells with ionophore or TPA (Figs. 3–5) was paralleled by an internalization of the intracellular domains of the PTPases, whereas the extracellular domains, as expected, could no longer be detected at the cell surface. EGTA-induced internalization was not paralleled by proteolytic processing of the PTPases and was therefore distinct from ionophore and TPA-induced internalization.

Surface Morphology of Intercellular Junctions

The localization of LAR and PTPσ at cell–cell contacts and their partial or complete internalization raised the question whether and how EGTA, ionophore, and TPA had affected the structural integrity of these specialized membrane regions. On scanning electron microscopy, untreated and vehicle (DMSO)-treated A431 cells were found to have formed multiple adhesion complexes between neighboring cells (Fig. 8, *A* and *B*). Incubation with EGTA (Fig. 8 *C*), as well as subsequent treatment with ionophore (Fig. 8 *D*) or ionophore alone (Fig. 8 *E*) induced an almost complete disruption of intercellular junctions, the formation of multiple surface protrusions, and a rounded elevation of the normally flat cell body from the surface. 40 min of treatment with TPA, which was also associated with internalization of LAR and PTPσ on immunolabeling studies (Fig. 7 *H*), left the junctional morphology completely intact (Fig. 8 *F*). These observations indicated that only the EGTA- and ionophore-induced internalization was paralleled by a structural disruption of intercellular adhesions, whereas TPA-induced internalization occurred independently of this effect.

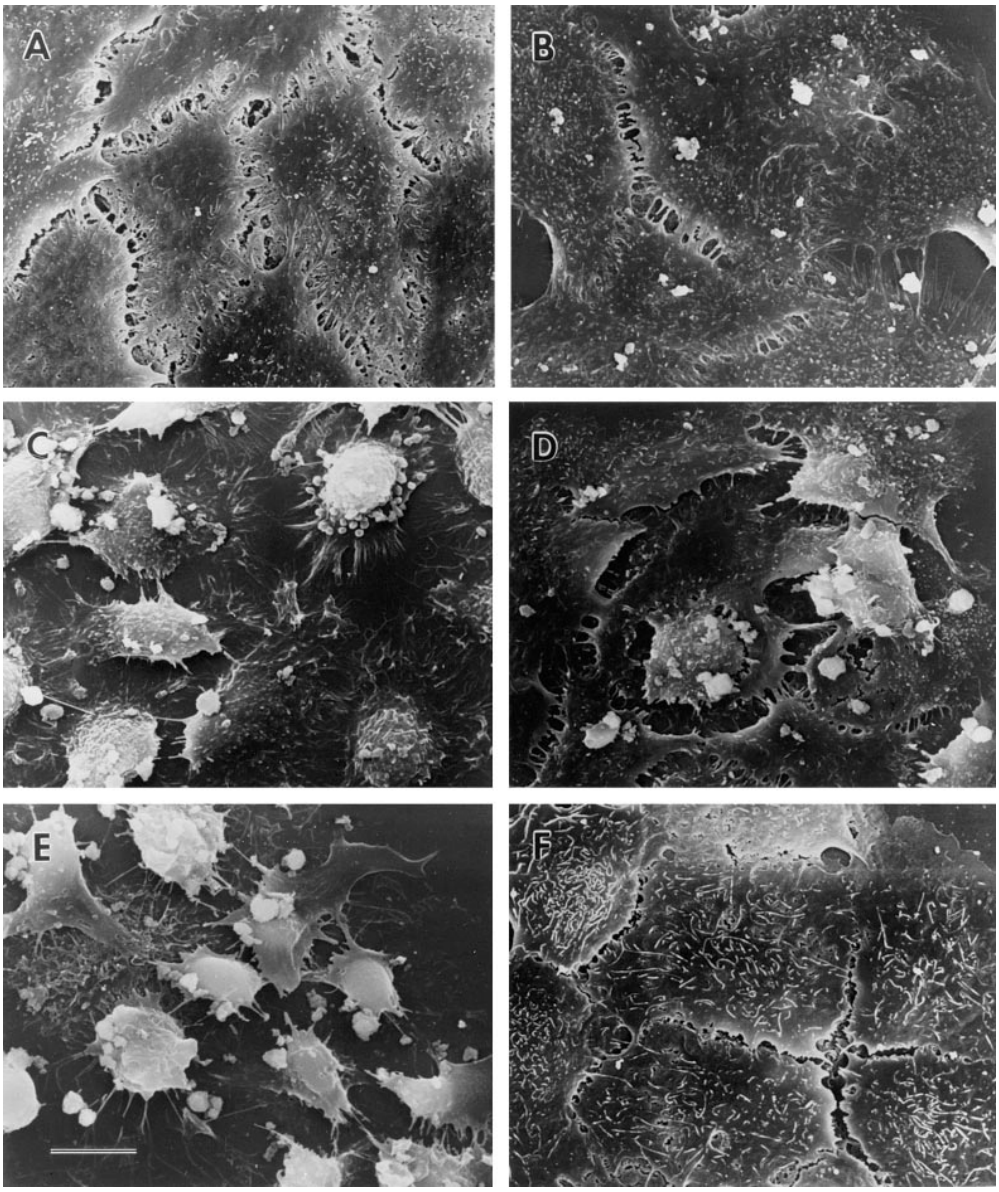

Figure 8. Surface morphology of intercellular adhesions. A431 cells were fixed and prepared for scanning electron microscopy as described in Materials and Methods. Prefixation treatment was as follows: (*A*) control; (*B*) vehicle (DMSO); (*C*) EGTA (5 mM) for 45 min; (*D*) EGTA (5 mM) for 45 min plus A23187 (10^{-5} M) for the last 30 min; (*E*) A23187 (10^{-5} M) for 30 min; (*F*) TPA (1 μM) for 40 min. Note intact cell–cell contacts in *A*, *B*, and *F* and largely disrupted intercellular junctions in *C*, *D*, and *E*. Bar, 10 μm.

Colocalization of LAR and PTPσ with Plakoglobin

We used antibodies directed against plakoglobin (γ-catenin), a protein localized at the intracellular site of adherens junctions and desmosomes (Cowin et al., 1986), to study whether LAR and PTPσ colocalize to these specialized areas and to investigate whether the internalization of the phosphatases from the plasma membrane is paralleled by a dissociation of plakoglobin from this site. Plakoglobin (Fig. 9 *A*) was detected along cell–cell contacts of neighboring A431 cells, strongly colocalized with antiserum 320 label (Fig. 9 *B*), and therefore identified the subcellular site of LAR and PTPσ as adherens junctions and desmosomes. Upon ionophore treatment, plakoglobin (Fig. 9 *C*) and the phosphatase domains were internalized in parallel from the rapidly dissociating intercellular junctions (Fig. 9 *D*). Incubation with TPA for 40 min left the localization of anti-plakoglobin labeling unaffected (Fig. 9 *E*) but induced a significant yet incomplete internalization of 320 label (Fig. 9 *F*). Extended time course experiments with TPA treatment up to 4 h showed that plakoglobin, in agreement with studies by Fabre and DeHerreros (1993), is redistributed from the plasma membrane to the cytosol (Fig. 9 *G*), although at a much slower rate and not in parallel with the intracellular phosphatase domain (Fig. 9 *H*). When antibodies directed against the extracellular domains of LAR and PTPσ were used for immunolabeling, both ionophore treatment and TPA incubation induced a complete disappearance of fluorescence label (data not shown).

These data indicated that LAR and PTPσ colocalized with plakoglobin to adherens junctions and desmosomes. The subsequent internalization of the intracellular phosphatase domains occurred rapidly and in parallel with plakoglobin after ionophore stimulation. TPA-induced internalization of plakoglobin occurred at much longer treatment intervals and not in parallel with that observed for the P subunits of the PTPases.

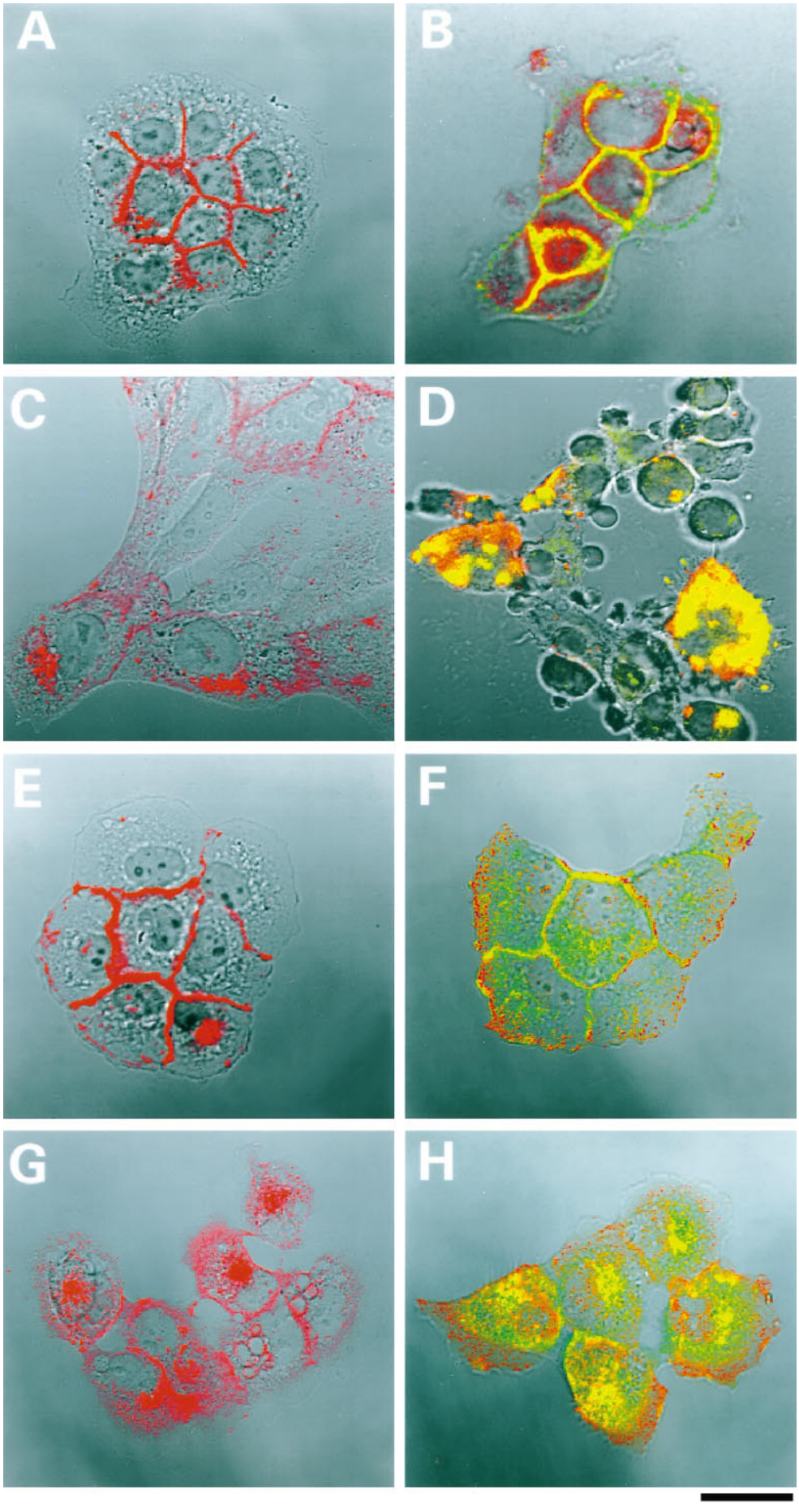

Figure 9. Colocalization of LAR and PTPσ with plakoglobin after ionophore and TPA treatment. A431 cells were fixed and labeled for plakoglobin (*A*, *C*, *E*, and *G*) or double labeled with anti-plakoglobin and antiserum 320 (COOH terminus of LAR and PTPσ; *B*, *D*, *F*, and *H*) as described in Materials and Methods. Laser confocal fluorescence images were superimposed on a transmission image to show protein localization together with cellular and junctional morphology. Red pseudocolor indicates monoclonal anti-plakoglobin antibody; green, polyclonal 320 antiserum; and yellow, regions of colocalization of both. Prefixation treatment was as follows: (*A* and *B*) controls; (*C* and *D*) A23187 (10^{-5} M) for 30 min; (*E* and *F*) TPA (1 μM) for 40 min; (*G* and *H*) TPA (1 μM) for 240 min. Note rapid internalization of label and disruption of cell–cell contacts in *C* and *D*. While 40 min of TPA neither disrupted intercellular junctions nor altered plakoglobin localization (*E*), antibody 320 label was already found in the cytosol (*F*). After 4 h of TPA treatment, significant plakoglobin label (*G*) and even more antibody 320 label (*H*) were found internalized in comparison to the label remaining at the plasma membrane or at cell–cell adhesions. Bar, 25 μm.

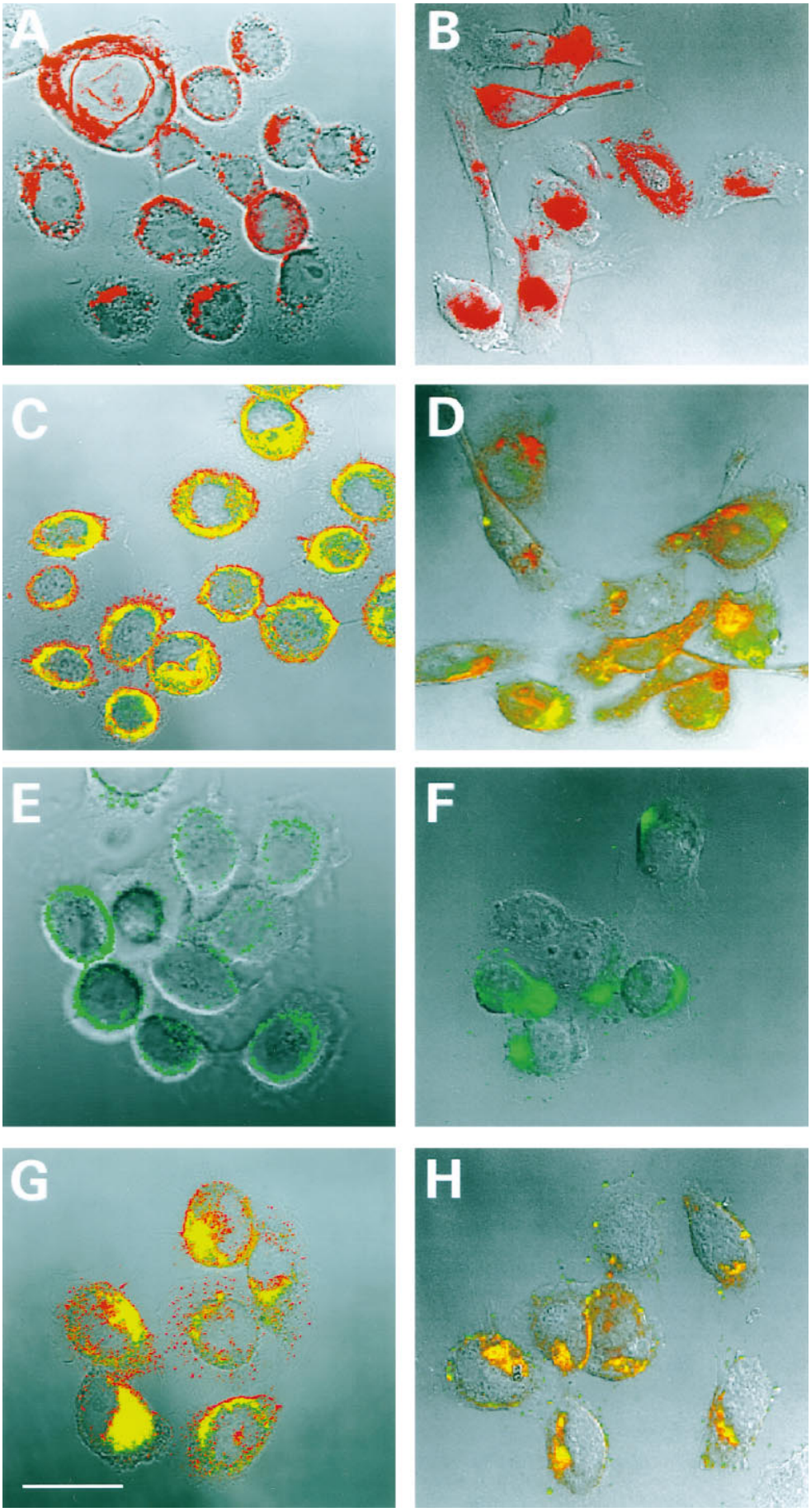

Figure 10. Colocalization of LAR and PTPσ with plakoglobin after EGTA treatment. Cells were prepared, labeled, and imaged as indicated in Fig. 9, legend. Prefixation treatment was EGTA (5 mM) for 45 min (*A*, *C*, *E*, and *G*) and EGTA (5 mM) for 240 min (*B*, *D*, *F*, and *H*). Cells were labeled with anti-plakoglobin antibody (*A* and *B*), anti-plakoglobin antibody plus antiserum 320 (*C* and *D*), antiserum αLAR_{EN} (NH_2 terminus of LAR; *E*), αLAR_{EN} plus anti-plakoglobin antibody (*G*), antiserum 322 (NH_2 terminus of PTPσ; *F*), and 322 plus anti-plakoglobin antibody (*H*). Note that EGTA treatment induces rapid disruption of intercellular junction and internalization of plakoglobin (*A* and *B*) together with the intracellular phosphatase domain (*C* and *D*) from the plasma membrane. EGTA also induces internalization of the extracellular E subunits of the PTPases (*E* and *F*), which can be colocalized with plakoglobin (*G* and *H*). Bar, 25 μm.

Localization of LAR and PTPσ after EGTA Treatment

The previous experiments demonstrated that the TPA-induced shedding of the E subunit and the internalization of the P subunit of LAR and PTPσ could occur in the presence of intact cell–cell adhesions. Whether, however, the disruption of cell–cell contacts is automatically associated with internalization of the P subunit remained unknown. We showed in Fig. 7, that EGTA induced a rapid and almost complete disruption of intercellular junctions, which should lead to the dissociation of protein complexes at these subcellular sites (Kartenbeck et al., 1991). Plakoglobin label in A431 cells was indeed found in the cytosol under the plasma membrane after 45 min of EGTA treatment (Fig. 10 *A*) and in the perinuclear cytosol after 4 h EGTA (Fig. 10 *B*). Colocalization with the intracellular domains of LAR and PTPσ demonstrated the same route of redistribution from the membrane to the cytosol (Fig. 10, *C* and *D*). Immunofluorescent staining with LAR and PTPσ antibodies directed against the extracellular domain alone (Fig. 10, *E* and *F*) or in colocalization with plakoglobin (Fig. 10, *G* and *H*) resulted in exactly the same appearance as observed for the intracellular domains of the PTPases. Shown here is the 45-min treatment for LAR (Fig. 10, *E* and *G*) and the 4-h treatment for PTPσ (Fig. 10, *F* and *H*). Immunofluorescent labeling at other time points was identical for both PTPases (data not shown).

In contrast to ionophore and TPA treatment, the disruption of intercellular junctions by EGTA did not induce shedding of the E subunits but instead led to an internalization of the intracellular as well as the extracellular part of LAR and PTPσ. The previously mentioned experiments showed that this redistribution occurred without prior proteolytic cleavage of LAR and PTPσ and could therefore involve the entire and intact molecule.

In Vitro Association of LAR with Proteins of the Cell Adhesion Complexes in Adherens Junctions and Desmosomes

To determine whether the PTPases not only localize to adherens junctions and desmosomes but can also associate with known proteins of the cell adhesion complexes at these sites, we performed in vitro association experiments. E-cadherin, α- and β-catenin, and plakoglobin were transiently expressed in 293 cells. Cells were incubated with or without the phosphatase inhibitor pervanadate before lysis to study the influence of tyrosine phosphorylation of these proteins on the association with LAR. Lysates were then incubated with glutathione-sepharose–bound GST-fusion protein, GST–hPTP-LAR_i, containing the entire intracellular domain of LAR, from amino acids 1,259 to 1,881. Bound proteins were analyzed by immunoblotting with antibodies directed against β-catenin (Fig. 11 *A*), plakoglobin (Fig. 11 *B*), α-catenin (data not shown), and E-cadherin (data not shown).

Association of β-catenin (Fig. 11 *A*) and plakoglobin (Fig. 11 *B*) with the GST-fusion protein, GST–hPTP-LAR_i, was detected in lysates of cells that expressed these proteins but was absent in controls. The amounts of associated proteins were independent of prior pervanadate treatment of the cells. In contrast, no specific association could be observed with lysates from α-catenin- or E-cadherin–expressing cells (data not shown), even though overexpression of these proteins in 293 cells was confirmed by immunoblotting of the same lysates with the appropriate antibodies (data not shown).

The observed, direct in vitro association between the intracellular domain of LAR with plakoglobin and β-catenin suggests that LAR not only colocalized with cell adhesion molecules at sites of cell–cell contact but that these proteins also interact in a functional manner. However, the question whether plakoglobin and β-catenin represent physiological targets of LAR could not be answered, because

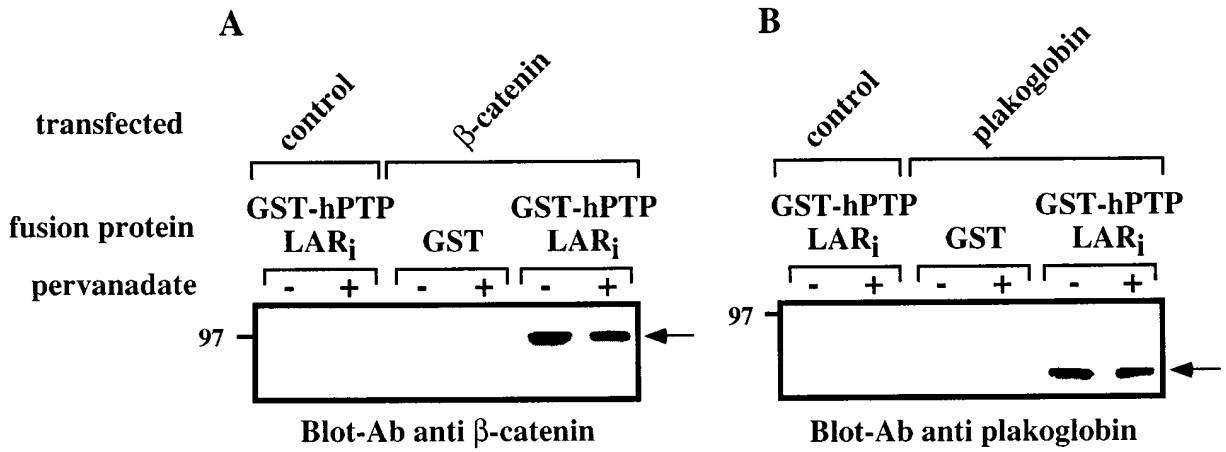

Figure 11. In vitro association of LAR with plakoglobin and β-catenin. β-catenin and plakoglobin were transiently expressed in 293 cells, and cells were stimulated for 10 min with pervanadate before lysis. Equal amounts of lysates were incubated with the LAR–GST-fusion protein, GST–hPTP LAR_i, or a threefold molar excess of GST, complexes were immobilized on glutathione–sepharose, and precipitates were separated by SDS-PAGE. Lysates of control plasmid-transfected 293 cells were bound in the same way to GST–hPTP LAR_i-glutathione–sepharose. Bound proteins were analyzed by immunoblotting with antibodies specific for β-catenin (*A*) or plakoglobin (*B*). Arrows indicate the proteins of interest; molecular size standards in kD are shown on the left.

under our experimental conditions the association was independent of tyrosine phosphorylation of these proteins.

Discussion

We compared structural and functional characteristics of the highly related PTPases LAR and PTPσ in regard to spontaneous as well as induced proteolytic processing. In analogy to LAR (Streuli et al., 1992; Yu et al., 1992) and other members of the type II class of PTPases (Jiang et al., 1993; Brady-Kalnay and Tonks, 1994; Pulido et al., 1995*a*; Fuchs et al., 1996), PTPσ was expressed in two subunits that were derived from a precursor protein by proteolytic processing. The similarities in the sequence as well as the structure among the members of this subfamily of PTPases suggest that they may also have closely related functions. This assumption is further supported by the observation that both (LAR and PTPσ) underwent analogous processing in response to intracellular calcium concentration increase or in response to treatment with TPA. Interestingly, while TPA-induced processing of overexpressed LAR required co-overexpression of PKCα in 293 cells, this was not necessary for PTPσ, suggesting differential effector sensitivity of the two PTPases. In 293 cells that overexpressed LAR or PTPσ as well as in cell lines that expressed these PTPases endogenously, the inducible processing took place at the NH_2 terminus of the P subunit removing a 14-kD fragment from LAR and an 8-kD fragment from PTPσ. It also resulted in shedding of the E subunits of both PTPases. By NH_2-terminal sequencing we identified the cleavage site of PTPσ and found it to be located between amino acids Pro_{821} and Ile_{822}, which corresponds to a distance of merely six amino acids from the transmembrane region. The analogous cleavage position of LAR does not show any sequence homology to that of PTPσ. Because the E subunit of LAR could be detected in the cellular supernatant after proteolytic processing, the cleavage of LAR also occurred extracellulary. Serra-Pages et al. (1994) have already demonstrated by mutational analysis that the cleavage of LAR in response to TPA occurred at a site that is located COOH-terminally to amino acid 1,222. Despite the absence of any sequence homology to the cleavage site of PTPσ, the proteolytic processing of LAR therefore occurred in a completely analogous position that is located between amino acids 1,222 and the first amino acid of the transmembrane region, Met_{1235}.

Solubilization of extracellular domains of transmembrane proteins by proteolytic processing has been observed for several transmembrane proteins (for review see Ehlers and Riordan, 1991). The exact cleavage sites, however, are in most cases not characterized. The transmembrane molecules that have been most thoroughly investigated are those growth factors that are released as soluble proteins by proteolytic processing from a transmembrane proprotein. The cleavage sites of these different growth factors also show no significant sequence homology but display clusters of small nonpolar amino acids (for review see Massague and Pandiella, 1993). The same enrichment of nonpolar amino acids was found in the cleavage position of PTPσ. While proteases responsible for the shedding of extracellular domains of transmembrane proteins have not been identified, a protease activity involved in the proprotein processing of TGFα had been characterized. It is a transmembrane protease whose active center was shown to be located in its extracellular part (Harano and Mizuno, 1994). Since the cleavage of the TGFα proprotein could be activated by TPA and A23187 (Pandiella and Massague, 1991), the same agents that we used to induce the processing of LAR and PTPσ, the transmembrane character of this protease can serve as a model for the mechanisms that allow intracellular signals to increase the extracellular activity of an enzyme. For the proprotein processing of TGFα, as opposed to the processing of the PTPases, the presence of a COOH-terminal valin residue in the short intracellular domain was an essential requirement (Bosenberg et al., 1992). While we cannot conclude from our study that the same or a related protease mediated the inducible processing of LAR and PTPσ, we excluded the possibility that the protease calpain, which mediated the processing of the intracellular PTPase 1B (Frangioni et al., 1993) was involved here. We therefore suggest that the processing of LAR and PTPσ underlies a different mechanism and is distinct from the processing of PTP1B.

The observation that the characteristics as well as the size of the resulting cleavage proteins were comparable in all the cell lines we studied indicated that the processing of LAR and PTPσ was a specific process and a ubiquitous event in cell lines of different origins. Moreover, the fact that a solubilization of the E subunits could also be observed for PTPμ (Gebbink et al., 1995) and PTPδ (Pulido et al., 1995*a*) suggested that this form of processing could be a general feature of type II PTPases. The cell density-dependent shedding of the E subunit of LAR has been proposed to play a role in the contact inhibition of cell growth (Streuli et al., 1992). The latter report and a study by Serra-Pages et al. (1994) showed that the shedding of the E subunits of PTPases in response to high cell density was caused by proteolytic processing. A cell density-dependent increase in the catalytic activity of PTPases was observed in contact-inhibited fibroblasts (Pallen and Tong, 1991) as well as in A431 cells (Mansbridge et al., 1992), while the phosphatase inhibitor vanadate was found to abolish the contact inhibition of rat kidney cells under certain experimental conditions (Rijksen et al., 1993). The underlying mechanism, however, through which the processing of transmembrane PTPases participates in cell contact inhibition is not yet understood. An increase of phosphatase expression with growing cell density has been observed for some PTPases (Longo et al., 1993; Östman et al., 1994; Celler et al., 1995; Fuchs et al., 1996). Cleavage and subsequent loss of the extracellular domains from receptor-type PTPases could also contribute to increased phosphatase activity. This hypothesis was supported by the observation that incubation of fibroblasts with trypsin, a treatment that permitted the cleavage of extracellular domains from transmembrane proteins, increased the phosphatase activity in these cells (Maher, 1993). However, in our study neither the in vitro activity of the processed P subunits of LAR and PTPσ nor their activity towards possible substrates, such as receptor tyrosine kinases, was found to be altered in cotransfection studies (data not shown).

To explore putative biological targets for the cleaved PTPases, we studied how the inducible processing would affect the subcellular localization of LAR and PTPσ and

used confocal microscopy in conjunction with cells that endogenously express both proteins. In untreated A431 cells, LAR and PTPσ were found at focal cell substratum adhesions (data not shown; Serra-Pages et al., 1995) as well as at sites of cell–cell contacts. The colocalization with plakoglobin (Cowin et al., 1986) identified these structures as adherens junctions and desmosomes. These subcellular compartments are known to contain cell adhesion molecules of the cadherin/catenin family, which mediate cell–cell adhesion by a homologous, calcium-dependent interaction of their extracellular domains and by linking the cell contact sites to the cytoskeleton via proteins bound to their intracellular portion (for reviews see Grunwald, 1993; Koch and Franke, 1994). Increased tyrosine phosphorylation at the sites of cell–cell and cell–substratum adhesions correlated with a number of biological processes such as cell migration, malignant transformation, and metastatic spread of tumor cells (for reviews see Kemler, 1993; Clark and Brugge, 1995; Rosales et al., 1995). The close association between cell contact disruption and malignant behavior actually led to the conclusion that cadherins represent a separate class of tumor suppressors (Birchmeier et al., 1995). We now show here that LAR and PTPσ colocalize with cell adhesion proteins at sites of cell–cell contact, an observation that suggests that LAR and PTPσ may be involved in either the formation or maintenance of intercellular junctions. This is further supported by the direct association of plakogobin and β-catenin with a GST-fusion protein of the intracellular domain of LAR in vitro. Although this association was found to be independent of tyrosine phosphorylation of plakogobin and β-catenin, we cannot rule out that phosphorylation plays a role for the in vivo interaction of PTPases with these proteins. An association of proteins of the cadherin/catenin complex was not only reported for PTPases of the LAR family (Kypta et al., 1996) but also for two other members of class II PTPases, PTPκ (Fuchs et al., 1996) and PTPμ (Brady-Kalnay et al., 1995). These two PTPases (Brady-Kalnay et al., 1995; Gebbink et al., 1995; Fuchs et al., 1996) and PCP-2 (Wang et al., 1996) also localized to sites of cell–cell contacts. However, the linkage of LAR to focal cell–substratum adhesions via interaction with the protein LIP-1 (Serra-Pages et al., 1995) and the association of PTPσ with LIP-1 (Pulido et al., 1995*b*) make it likely that the supposed regulation of cadherin/catenin complexes is not the only function of LAR and PTPσ.

When we analyzed the consequences of proteolytic processing on the subcellular localization of LAR and PTPσ we found that the treatment with either TPA or A23187 resulted in a loss of labeling for the extracellular domain. This finding was in complete agreement with the cleavage and shedding of the E subunits of both PTPases, which we observed in biochemical experiments. The effect, however, that these agents showed on the localization of the shortened P subunit of LAR and PTPσ was quite different. A23187, while inducing a complete disruption of cell–cell contacts within minutes, led to an even cytosolic distribution of the P subunits and a loss of labeling at the cell membrane. A brief treatment with TPA, on the other hand, left cell–cell contacts morphologically intact but reduced the concentration of P subunits at these cell–cell contacts by inducing only a partial internalization of label to the cytosol. After longer treatment intervals with TPA, a structural disturbance of cell–cell adhesions was finally observed and was paralleled by a greater internalization of P subunits. Interestingly, a depletion of calcium in the culture medium with EGTA also resulted in an internalization of both PTPases. This effect, however, occurred independent of proteolytic processing, was not associated with a shedding of E subunits, and involved both subunits of the presumably intact PTPases.

Based upon these observations, we conclude that at least two independent mechanisms for the internalization of LAR and PTPσ exist. One of them involves the induction of proteolytic processing, which, as demonstrated in the TPA experiments, can lead to internalization of P subunits even in the presence of intact cell–cell contact. The other mechanism involves the disruption of cell–cell contact but, as shown in the EGTA experiments, can lead to internalization of the intact PTPases without prior proteolytic processing.

The proteins of the cadherin/catenin complexes at focal cell–cell contacts underwent a very similar internalization and, later, degradation when cells were treated with EGTA (Kartenbeck et al., 1991) or TPA (Fabre and De Herreros, 1993). However, the time course of internalization for plakoglobin and the PTPases differed in our experiments and were dependent on the agent used. Whereas short-time treatment with TPA left the integrity of intercellular junctions and the localization of plakoglobin unaffected, it already induced a significant yet incomplete internalization of the PTPases. At longer periods of incubation, intercellular junctions became more and more disrupted, and plakoglobin was also redistributed from the plasma membrane to the cytosol, but not in parallel and at a much slower rate than the intracellular phosphatase domains. In contrast to the effect observed with TPA, ionophore and EGTA, two agents that disrupted cell–cell contacts, induced a rapid and simultaneous internalization of both plakoglobin and the intracellular PTPase domains. We conclude from these data that the agents that we have shown to disrupt intercellular junctions simultaneously induce the internalization of LAR and PTPσ and disturb the integrity of the cadherin/catenin complexes at the same subcellular sites. With TPA, on the other hand, the internalization of the PTPases occured before the integrity of cellular junctions was affected, and the loss of the PTPases at these subcellular sites presumably contributes to the later disruption of cell–cell contacts.

LAR and PTPσ did not only colocalize with cell adhesion proteins of the cadherin/catenin family and were subjected to a comparable intracellular redistribution under experimental conditions, but also their extracellular domains were shed in a manner that was also reported for cadherins (Wheelock et al., 1987; Roark et al., 1992). Interestingly, the soluble extracellular domain of N-cadherin, NCAD90, remained capable of binding N-cadherin in a homophilic manner and promoted cell adhesion and neurite growth in chicken embryo retina cells when presented in a substrate-bound form (Paradies and Grunwald, 1993). Moreover, the soluble extracellular domain of E-cadherin was able to inhibit cell adhesion (Wheelock et al., 1987). It is therefore possible that the extracellular domains of receptor-type PTPases, which are also cleaved

from the cell surface upon proteolytic processing, could be equally involved in the formation, maintenance, or restoration of cell adhesions. While the distinct subcellular redistribution of presumably catalytically active P subunits of LAR and PTPσ under normal conditions may regulate proliferation and tissue integrity by cell–cell contact through targeting the phosphatase activity to specific intracellular sites, the biological function of the solubilized E subunits remains at present unknown. Under pathophysiological conditions such as malignant cell transformation, the regulatory mechanism investigated here may represent a critical target for subversion leading to dysregulated growth and metastatic spread.

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