The EMBO Journal vol.11 no.3 pp.897-907, 1992

Expression of the receptor-linked protein tyrosine phosphatase LAR: proteolytic cleavage and shedding of the CAM-like extracellular region

Michel Streuli[1,2], Neil X.Krueger[1,2], Pamela D.Ariniello[5], May Tang[1], J.Michael Munro[1,2], Walter A.Blattler[5], David A.Adler[4], Christine M.Disteche[4] and Haruo Saito[1,3]

[1]Division of Tumor Immunology, Dana-Farber Cancer Institute, 44 Binney Street, Boston, MA 02115, Departments of [2]Pathology, and [3]Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, MA 02115, [4]Department of Pathology, University of Washington, Seattle, WA, 98195 and [5]ImmunoGen Inc., Cambridge, MA 02139, USA

Communicated by C.Weissmann

The human transmembrane molecule LAR is a protein tyrosine phosphatase (PTPase) with a cell adhesion molecule-like extracellular receptor region. The structure of LAR hinted at its involvement in the regulation of tyrosine phosphorylation through cell-cell or cell-matrix interactions. We show here that LAR is expressed on the cell surface as a complex of two non-covalently associated subunits derived from a proprotein. The LAR E-subunit contains the cell adhesion molecule-like receptor region, while the LAR P-subunit contains a short segment of the extracellular region, the transmembrane peptide and the cytoplasmic PTPase domains. Proprotein processing occurs intracellularly. Analysis of LAR mutants suggested that cleavage occurs in the LAR extracellular region at a paired basic amino acid site by a subtilisin-like endoprotease. A single amino acid substitution at this site blocked LAR proprotein cleavage. The LAR E-subunit is shed during cell growth, suggesting that LAR receptor shedding may be a mechanism for regulating PTPase function. The use of immunohistochemistry techniques on human tissues demonstrated the expression of LAR by various cell lineages, including epithelial cells, smooth muscle cells and cardiac myocytes. The *LAR* gene is mapped to chromosome 1, region p32-33, which contains candidate tumor suppressor genes.

Key words: human chromosome 1/immunohistochemistry/paired basic amino acid site/protein tyrosine phosphorylation

Introduction

Protein tyrosine phosphorylation plays an essential role in regulating cell proliferation. The recent identification and characterization of a family of cytosolic and receptor-linked protein tyrosine phosphatases (PTPases) suggests that the dephosphorylation, as well as the phosphorylation by protein tyrosine kinases, of key signal transduction proteins may be regulated by extracellular signals and that PTPases probably have substrate and tissue specificities (reviewed by Hunter, 1989; Saito and Streuli, 1991; Fischer *et al.*, 1991; Bishop, 1991). Thus, PTPases may be important in the regulation of cell proliferation. Furthermore, given that hyperphosphorylation of protein tyrosine residues can cause cell transformation, it is plausible that lack of dephosphorylation resulting from loss of a PTPase function may also be oncogenic. Indeed, the human gene *PTPγ*, which encodes a receptor-linked PTPase, maps to the chromosomal region 3p21 (LaForgia *et al.*, 1991). This region is frequently deleted in renal cell and lung carcinomas, suggesting that the *PTPγ* gene in particular and PTPase genes in general, may be candidate tumor suppressor genes.

To date, at least 14 human PTPases have been identified, all of which contain one or two homologous PTPase domains (Saito and Streuli, 1991). These PTPase domains are ~300 amino acids long and contain ~40 highly conserved amino acid positions. Typically, any two individual PTPase domains are 30-50% identical. Six of these PTPases, PTP-1B (Charbonneau *et al.*, 1988; Brown-Shimer *et al.*, 1990; Chernoff *et al.*, 1990; Guan *et al.*, 1990), TC-PTP (Cool *et al.*, 1989), PTP-MEG (Gu *et al.*, 1991), PTPH1 (Yang and Tonks, 1991), STEP (Lombroso *et al.*, 1991) and PTP1C (Shen *et al.*, 1991) are cytosolic enzymes with only one PTPase domain. The other eight human PTPases, leukocyte common antigen [LCA/CD45 (reviewed by Thomas, 1989)], LAR (Streuli *et al.*, 1988a), PTPα, β, γ, δ, ε and ζ (Krueger *et al.*, 1990; Kaplan *et al.*, 1990; Matthews *et al.*, 1990; nomenclature according to Saito and Streuli, 1991) are all transmembrane proteins with extracellular regions connected to cytoplasmic PTPase domains by a transmembrane peptide. The extracellular regions of these proteins are in many cases distinct in size and sequence, while the cytoplasmic region sequences are all similar because of their PTPase domains. All of the receptor-linked PTPases contain two PTPase domains, except HPTPβ which has only one PTPase domain. Site-directed mutagenesis studies have shown that one conserved cysteine residue, located in the transmembrane proximal PTPase domain of both LCA and LAR, is absolutely required for PTPase activity and that several highly conserved amino acid positions surrounding the critical cysteine residue may be directly involved in the catalytic process (Streuli *et al.*, 1989, 1990). Furthermore, although the second (transmembrane-distal) PTPase domain of LAR and LCA does not appear to have catalytic activity, it may have a regulatory function (Streuli *et al.*, 1990).

Among the receptor-linked PTPases, LAR is of particular interest because its extracellular region resembles that of cell adhesion molecules (CAMs). The LAR extracellular region is composed of three Ig domains and eight FN-III domains (Streuli *et al.*, 1988a). Ig domains are thought to function as cell surface recognition structures and are found in several growth factor receptors such as the PDGF, FGF and IL-6 receptors and in adhesion molecules, including the neural-CAM (N-CAM) (Williams and Barclay, 1988). FN III domains are ~90 amino acids long and contain a

characteristic sequence motif identified initially in fibronectin (Patthy, 1990). The combination of Ig and FN III domains is found in a number of known CAMs and also in the putative tumor suppressor *DCC* (deleted in colorectal cancers) gene product (Fearon *et al.*, 1990). The CAM-like extracellular region of LAR suggests that it is a cell adhesion receptor and that LAR PTPase activity is regulated by interactions at the receptor end of the molecule.

Although there has been considerable progress in determining the structures and enzymatic properties of PTPases, very little is known about the biosynthesis, regulation and tissue distribution of the receptor-linked PTPases, with the exception of the hematopoietic lineage-specific LCA PTPase. We have now analyzed the biosynthetic and tissue expression of the LAR PTPase using anti-LAR monoclonal antibodies (mAbs) and have shown that LAR is proteolytically processed intracellularly into two subunits that are non-covalently associated on the cell surface. By mutational analysis, a single amino acid residue was identified that is required for such proteolytic processing. Furthermore, the LAR extracellular subunit can be released from the cell surface, suggesting that LAR function may be regulated by receptor shedding. Immunohistochemical analysis demonstrated that LAR is expressed by various cell types, such as epithelial and smooth muscle cells, in a broad range of tissues. The *LAR* gene was mapped to chromosome 1, in a region that contains candidate tumor suppressor genes.

Results

Anti-LAR monoclonal antibodies

LAR is a transmembrane protein with an extracellular region composed of three Ig domains and eight FN-III domains connected to the cytoplasmic PTPase domains via a transmembrane peptide (Figure 1). Anti-LAR mAbs against the extracellular region of LAR were generated by immunizing mice and screening hybridomas with transfected murine cell lines that expressed either the human LAR protein or a hybrid LAR−LCA protein. The hybrid LAR−LCA protein contained the three Ig domains of LAR embedded in the N-terminal region of the 180 kDa LCA isoform (Figure 1). Epitope specificities of the mAbs were determined using the transfected murine cell lines expressing LAR, hybrid LAR−LCA or LCA. mAbs that bound to both the LAR−LCA hybrid and LAR were specific to the LAR Ig domains (38.1B, 75.3A and 108.4A), while antibodies that bound only to LAR were specific to the LAR FN III domains (11.1A, 71.2E, 128.4A and 136.1).

Cell surface LAR is composed of two subunits

To characterize LAR biosynthesis, we initially used the epitheloid carcinoma HeLa cell line because it expressed a relatively large amount of LAR by FACS analysis. Cell surface proteins of intact HeLa cells were ^{125}I-labelled, and cell lysates were prepared using NP-40 detergent. LAR proteins were immunoprecipitated from the lysates using three anti-LAR mAbs with distinct epitope specificities (128.4A, 11.1A and 75.3A) and resolved by SDS−PAGE (Figure 2A, lanes 2−4). The major protein immunoprecipitated by the three anti-LAR mAbs had an apparent molecular weight (M_r) of 150 000. This result was unexpected as the LAR cDNA encodes a putative protein of 1881 amino acids which should have an M_r of at least 200 000 (Streuli *et al.*, 1988a). In contrast to the single protein detected by cell surface iodination, anti-LAR immunoprecipitates from lysates made from HeLa cells that were metabolically labelled with [^{35}S]methionine, all revealed the existence not only of a 150 kDa protein but also of an 85 kDa protein (Figure 2B, lanes 1−4). It is unlikely that the 150 kDa and 85 kDa proteins were fortuitously cross-reacting with the LAR mAbs, because all three anti-LAR mAbs recognized the same protein.

To determine the relationship between the 150 kDa and 85 kDa LAR proteins, we performed pulse−chase experiments. HeLa cells were metabolically labelled with [^{35}S]-methionine for 15 min and chased with an excess of non-radioactive methionine for 0, 1, 2 or 4 h. Analysis of the anti-LAR immunoprecipitates revealed the existence of an ~200 kDa precursor protein immediately after the 15 min pulse period (Figure 2C, lane 2); this was chased into the 150 kDa and 85 kDa proteins within 1 h (Figure 2C, lanes 3−5). Pretreatment of cells with the antibiotic tunicamycin, which inhibits addition of N-linked oligosaccharides at asparagine residues of proteins, resulted in a LAR precursor protein of ~190 kDa (Figure 2C, lane 6) and products of ~140 kDa and 85 kDa (Figure 2C, lane

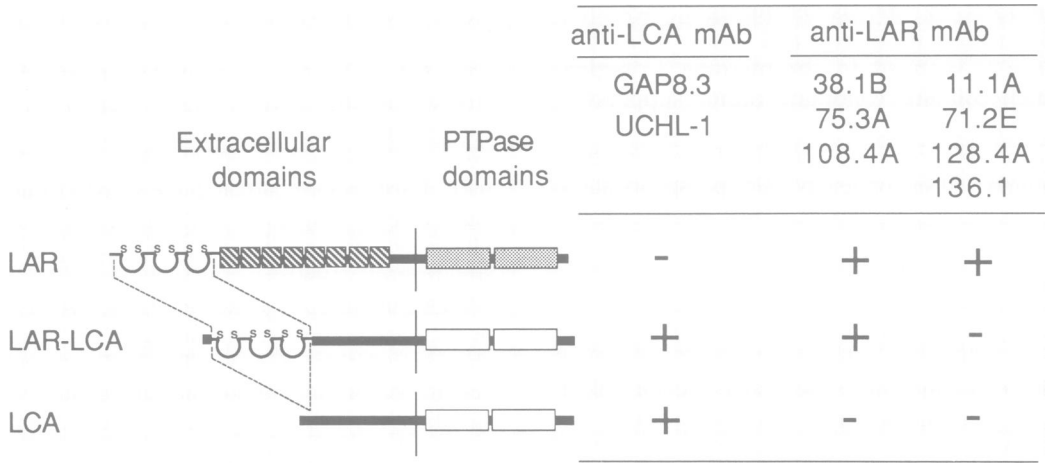


Fig. 1. Anti-LAR monoclonal antibody specificities. Schematically shown are the LAR, LCA and LAR−LCA hybrid proteins expressed in murine 300-19 cells and the reactivity (+) or lack of reactivity (−) of the indicated mAb to the three proteins. The LAR immunoglobulin-like, FN-III and intracytoplasmic PTPase domains are represented by characteristic disulfide-bonded structures, small hatched boxes and large stippled boxes, respectively. LAR−LCA contains the three Ig domains of LAR embedded in the N-terminal region of the 180 kDa LCA isoform.

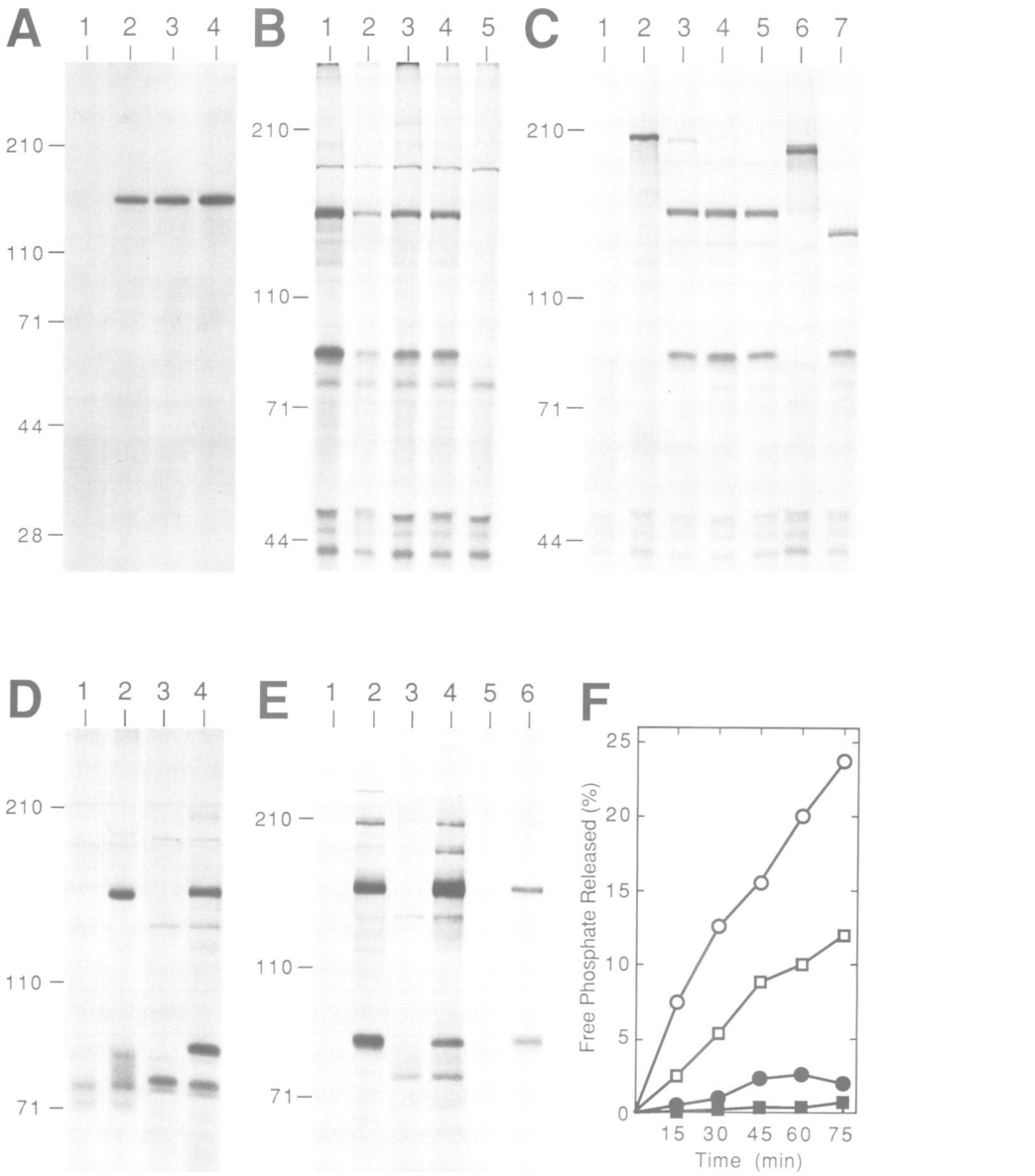
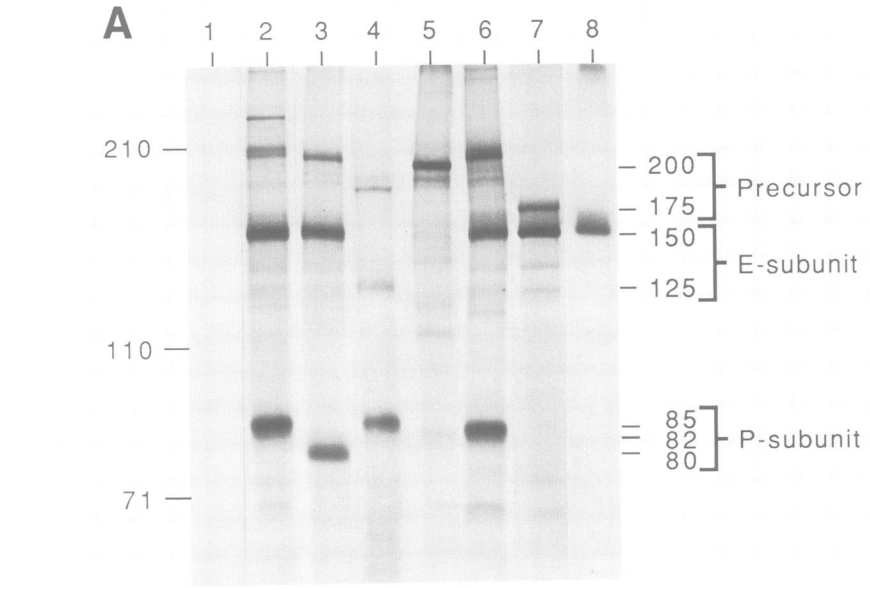
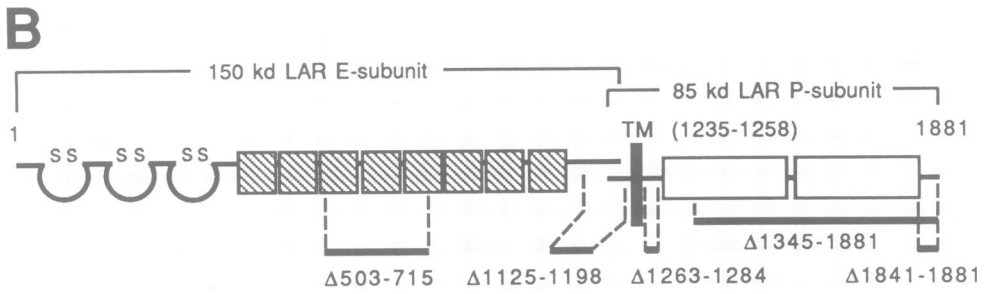


Fig. 2. Characterization of LAR biosynthesis. (**A**) SDS−PAGE of anti-LAR immunoprecipitates from ^{125}I-labelled HeLa cell lysates using individual anti-LAR mAb 128.4A (lane 2), 11.1A (lane 3) or 75.3A (lane 4), or control isotype matched mAb (lane 1). Molecular mass standards in kilodaltons are shown at left of figures. (**B**) SDS−PAGE of anti-LAR immunoprecipitates from [^{35}S]methionine-labelled HeLa cell lysates using individual anti-LAR mAb 128.4A (lane 2), 11.1A (lane 3), 75.3A (lane 4), a 1:1:1 mix of the mAb 128.4A, 11.1A and 75.3A (anti-LAR mix; lane 1), or with control isotype matched mAb (lane 5). (**C**) Pulse−chase analysis of LAR synthesis. HeLa cells were metabolically pulse-labelled with [^{35}S]methionine for 15 min and then chased with an excess of non-radioactive methionine for 0 h (lanes 2 and 6), 1 h (lane 3), 2 h (lanes 4 and 7) or 4 h (lane 5) before cell solubilization. Prior to pulse labelling, some of the cells were preincubated for 1 h with (20 μg/ml) tunicamycin (lanes 6 and 7). Anti-LAR (lanes 2−7) or control immunoprecipitates (lane 1) were analyzed by SDS−PAGE. (**D**) SDS−PAGE analysis of anti-LAR immunoprecipitates from [^{35}S]methionine-labelled HeLa cell lysates without reduction (lane 2) or with reduction (lane 4). Reduced and non-reduced samples on the SDS−PAGE gel were originally separated by eight lanes that did not contain 2-mercaptoethanol. Control immunoprecipitates using isotype matched mAb are shown in lanes 1 and 3. (**E**) SDS−PAGE analysis of anti-LAR immunoprecipitates using [^{35}S]methionine-labelled cell lysates from a murine B cell line expressing human LAR cDNA [300-19 (LAR); lane 2], the human epidermoid carcinoma cell line A431 (lane 4) and normal human epidermal keratinocytes (lane 6). Control immunoprecipitates using isotype matched mAb are shown in lanes 1, 3 and 5. (**F**) Protein tyrosine phosphatase activity of anti-LAR immunoprecipitates. LAR protein was immunoprecipitated from HeLa cell lysate (open circle) or 300-19 (LAR) cell lysate (open square) using the anti-LAR mAb mix, and immunoprecipitates were assayed using [^{32}P]Tyr-Raytide. PTPase activity from control immunoprecipitates, using isotype-matched mAb, from HeLa lysates (closed circle) or 300-19 (LAR) lysates (closed squares) are also shown. The amount of radioactivity released as inorganic phosphate is expressed as the percentage of total input radioactivity.

7). These data demonstrated a precursor−product relationship between the ~200 kDa protein and the 150 kDa and 85 kDa proteins. This precursor protein must, however, be expressed only intracellularly because it was not detected following ^{125}I-labelling of cell surface proteins. Furthermore, the data indicated that the N-linked oligosaccharide present on the ~200 kDa protein was chased into the 150 kDa protein. The pulse−chase data also excluded the possibility that the 150 kDa and 85 kDa LAR proteins were generated by non-specific proteolysis during sample preparation, because the cells lysed immediately following pulse labelling contained only the ~200 kDa protein. As





LAR cDNA	Lane	E-subunit	P-subunit	Precursor
wt (1-1881)	2	150 kd	85kd	
Δ503-715	4	125 kd	85 kd	
Δ1125-1198	5	ND	ND	~200 kd
Δ1263-1284	6	150 kd	82 kd	
Δ1345-1881	7, 8	150 kd	(25 kd)	175 kd
Δ1841-1881	3	150 kd	80 kd	

Fig. 3. Identification of the LAR E- and P-subunit structure by deletion analysis. (**A**) SDS−PAGE analyses of anti-LAR immunoprecipitates from [^{35}S]methionine-labelled (lanes 2−7), or ^{125}I-labelled (lane 8), 300-19 cell lines expressing wild-type LAR (lane 2) or mutant LAR Δ1184−1181 (lane 3), LAR Δ503−715 (lane 4), LAR Δ1125−1198 (lane 5), LAR Δ1263−1284 (lane 6) or LAR Δ1345−1881 (lanes 7 and 8). Control immunoprecipitates using isotype matched mAb are not shown except from [^{35}S]methionine-labelled 300-19 (LAR) cells (lane 1). Molecular mass standards in kilodaltons are shown at the left of the figure. At the right of the figure are indicated the positions and calculated M_r of the LAR E-subunit, P-subunit and the LAR precursor proteins. (**B**) Summary of immunoprecipitation analysis of LAR deletion mutations. At the top of the figure is schematically shown the structure of the LAR E- and P-subunits with the approximate site of proprotein cleavage. Positions and ranges of the deletions are shown immediately below the drawing. Below are listed the LAR cDNA with the various deletions that were expressed in 300-19 cells, the corresponding lane number(s) in panel A, and the M_r of the LAR subunits of precursor proteins encoded by the mutant LAR cDNA as determined by immunoprecipitation in A. TM, transmembrane; wt, wild-type LAR cDNA; ND, not detected.

SDS−PAGE analysis does not adequately resolve large proteins, it is possible that the ~200 kDa protein has an actual M_r of ~235 kDa, which is the sum of 150 kDa and 85 kDa.

To examine if the 150 kDa and 85 kDa proteins were associated by disulfide bonding, anti-LAR immunoprecipitates were analyzed without or with reduction by 2-mercaptoethanol (2-ME) (Figure 2D, lanes 2 and 4). Under non-reducing conditions, the mobilities of the 150 kDa and 85 kDa proteins were the same as under reducing conditions except that the 85 kDa protein migrated more diffusely. Therefore, the 150 kDa and 85 kDa proteins are not disulfide bonded. However, the association of the 150 kDa and 85 kDa subunits is sufficiently strong to resist disruption during vigorous washing of the immunoprecipitates.

LAR proteolytic processing was not a peculiarity of HeLa cells, because anti-LAR immunoprecipitates from [^{35}S]-methionine-labelled murine B cell line transfected with the

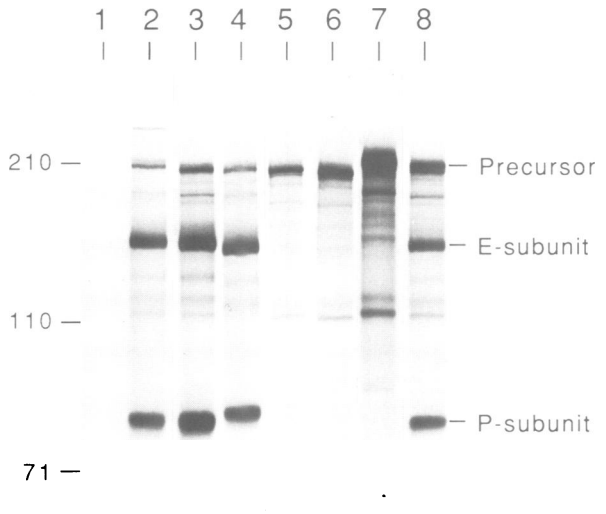


Fig. 4. Effect of amino acid substitutions of paired basic amino acids on LAR proprotein processing. SDS−PAGE analysis of anti-LAR immunoprecipitates from [^{35}S]methionine-labelled 300-19 cell lines expressing wild-type LAR (lane 2), or LAR proteins harboring the amino acid substitutions KK(1177−1178) → QL (lane 3), KR(1209−1210) → QL (lane 4), RRRRR(1148−1152) → RANNN (lane 5), RRRRR(1148−1152) → RARNR (lane 6), RRRRR(1148−1152) → RARRR (lane 7) or RRRRR(1148−1152) → RRRAR (lane 8). LAR amino acid substitution mutations are designated with the wild-type amino acid sequence at the left, the amino acid positions in parenthesis and the mutated sequence at the right (R: arginine, A: alanine, N: asparagine, Q: glutamine). Control immunoprecipitates using isotype matched mAb are not shown except for the control immunoprecipitate from 300-19 (LAR) cells (lane 1). Molecular mass standards in kilodaltons are shown at left. At the right of the figure are indicated the positions of the LAR E-subunit, P-subunit and the LAR precursor proteins.

LAR cDNA [300−19 (LAR)], human epidermoid carcinoma A431 cells and normal human epidermal keratinocytes (NHEK), all contained the 150 kDa and 85 kDa LAR proteins (Figure 2E, lanes 2, 4 and 6). Anti-LAR immunoprecipitates from 300-19 (LAR), A431 and NHEK cells also contained a protein of ~200 kDa, which was probably the LAR precursor protein. Furthermore, the finding that 300−19 (LAR) cells expressed the 150 kDa and 85 kDa proteins excluded the possibility that these two proteins were derived by alternative splicing, because the 300-19 (LAR) cells expressed a cDNA-derived LAR.

If anti-LAR mAbs specifically immunoprecipitate LAR, the precipitates should contain phosphotyrosine-specific phosphatase activity. The latter was confirmed by assaying the PTPase activity of the immunoprecipitates using phosphotyrosine−Raytide substrate (Streuli *et al.*, 1990). While control immunoprecipitates from HeLa or 300-19 (LAR) cell lysates using isotyped-matched mAb did not have significant PTPase activity, anti-LAR immunoprecipitates contained PTPase activity (Figure 2F). Overall the data in Figure 2 suggested that the precursor LAR protein is intracellularly cleaved into two subunits that remain associated as a heterodimeric structure, with the 150 kDa protein containing the N-terminus and the 85 kDa protein containing the C-terminus.

To confirm that the 150 kDa and 85 kDa proteins were the extracellular and cytoplasmic regions of LAR, respectively, we generated cDNA mutants that encoded LAR proteins with deletions in either the extracellular or cytoplasmic regions. LAR cDNA containing mutations that removed either 41 amino acids from the carboxy terminus (Δ1841−1881), 537 amino acids from the carboxy terminus (Δ1345−1881) or 213 amino acids of the LAR extracellular region (Δ503−715) were transfected into 300−19 murine B cells and resulting cell lines expressing cell surface LAR were identified by FACS analysis. LAR expressing cells were also metabolically labelled with [^{35}S]methionine or surface labelled with ^{125}I and immunoprecipitations using anti-LAR mAbs were carried out (Figure 3A). As summarized in Figure 3B, deletion of 41 amino acids from the carboxy-terminus (Δ1841−1881) resulted in a shift in M_r of the 85 kDa protein to 80 kDa, while the M_r of the 150 kDa protein was not affected (Figure 3A, lane 3). The 537 amino acid carboxy-terminal deletion (Δ1345−1881) also did not affect the M_r of the 150 kDa protein, but resulted in the apparent loss of the 85 kDa protein (this deletion yielded a protein of only ~25 kDa, which ran off the gels; Figure 3A, lane 7). The cell line expressing LAR Δ1345−1881 also contained a substantial amount of a protein having an M_r of 175 kDa (Figure 3A, lane 7). When these cells were ^{125}I-labelled, only the 150 kDa protein was labelled (Figure 3A, lane 8), suggesting that the 175 kDa protein was the cytoplasmically localized precursor protein. The deletion within the extracellular LAR region (Δ503−715) resulted in a decrease in the M_r of the 150 kDa protein to 125 kDa, while not affecting the M_r of the 85 kDa protein (Figure 3A, lane 4). These results demonstrate that the 150 kDa protein (designated LAR E-subunit) is indeed the extracellular portion of LAR and that the 85 kDa protein (designated LAR P-subunit) contains the intracytoplasmic PTPase domains and probably the transmembrane segment (Figure 3B).

Cleavage of the LAR proprotein requires a paired basic amino acid site

As the 85 kDa LAR P-subunit contains the C-terminal region and the M_r of the LAR cytoplasmic region alone (623 amino acids) is ~72 000, it is likely that the cleavage site lies within 150 amino acids of the extracellular region immediately adjacent to the transmembrane peptide. To test this possibility, we constructed an LAR cDNA (Δ1125−1198) that deleted 74 amino acids in the extracellular region preceding the transmembrane peptide; when this was expressed in murine B cells, there was no surface expression of LAR as determined by FACS analysis (data not shown). However, immunoprecipitation of [^{35}S]methionine-labelled proteins from transfected cell lines revealed that the ~200 kDa precursor LAR protein accumulated in the cytoplasm (Figure 3A, lane 5). In contrast, an LAR deletion that removes 22 amino acids of the cytoplasmic region immediately following the transmembrane peptide (Δ1263−1284) did not affect LAR processing (Figure 3A, lane 6); or LAR surface expression as determined by FACS analysis. Therefore, processing probably occurs in the LAR extracellular region and the subregion 1125−1198 contains important amino acids required for LAR processing and/or cell surface transport.

Because paired basic amino acid sequences are proteolytic cleavage sites for a number of proproteins (Barr, 1991), including the insulin receptor proprotein, we altered each of the paired basic amino acid sequences within the stretch of amino acids (1125−1198), which were shown to affect LAR precursor processing, by site-directed mutagenesis.

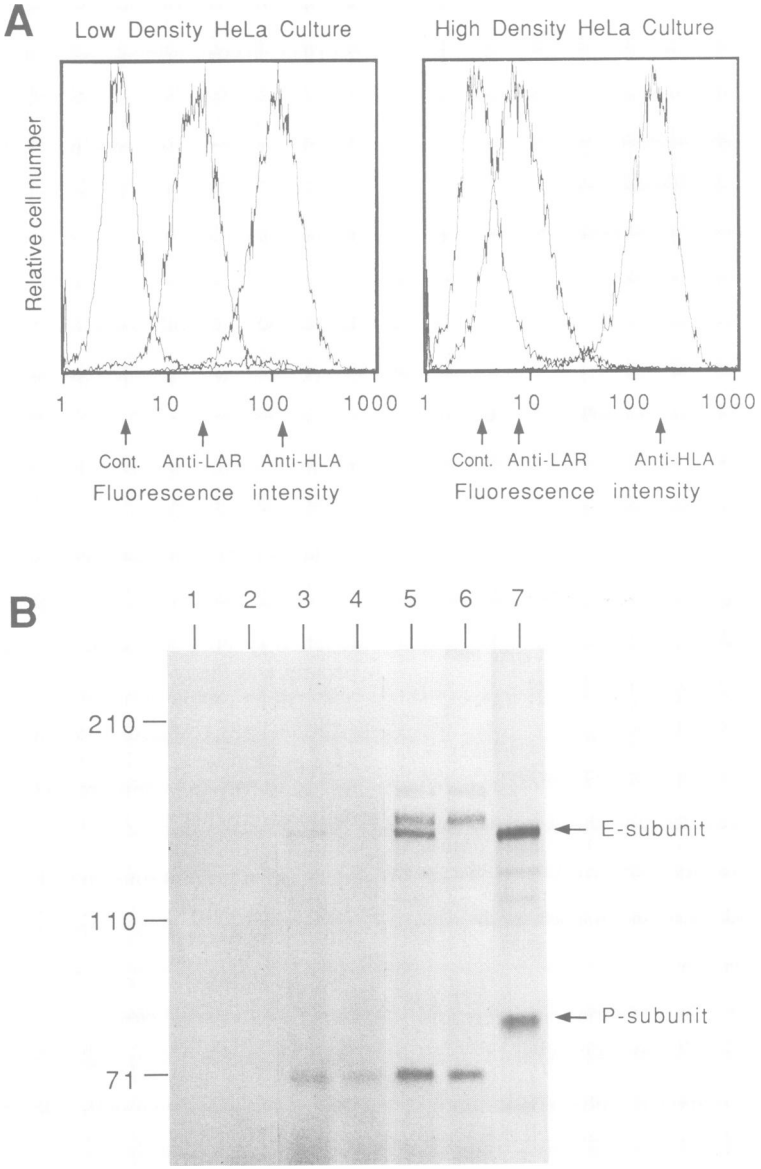


Fig. 5. Modulation of LAR E-subunit expression. (**A**) Flow cytometric analysis of cell surface LAR expression on low and high density HeLa cell cultures. HeLa cells were grown to a cell density of 4×10^4 cells/cm^2 (low density) or 3×10^5 cells/cm^2 (high density), harvested and analyzed as described in Materials and methods. 1×10^6 cells were stained using either the anti-LAR mAb 75.3A, the anti-HLA mAb W6/32 or control antibody. (**B**) SDS–PAGE analysis of anti-LAR immunoprecipitates from cell culture supernatants following pulse–chase labelling for 0 (lane 1), 1 (lane 3) or 6 h (lane 5), or from a cell lysate following pulse–chase labelling for 6 h (lane 7). Confluent HeLa cells ($\sim 1.5 \times 10^5$ cells/cm^2) were metabolically pulse-labelled with [^{35}S]methionine for 15 min and then chased with an excess of non-radioactive methionine for 0, 1, or 6 h. Cell culture supernatants were harvested following the chase period. Control immunoprecipitates from the 0, 1 or 6 h supernatants are shown in lanes 2, 4 and 6. The expected positions of the LAR 150 kDa E-subunit and 85 kDa P-subunit are indicated on the right. Molecular mass standards in kilodaltons are shown at left. The exposure time for the autoradiogram shown in lanes 1–6 was 26 days, and for lane 7, 72 h.

Within this region there is a lysine–lysine (KK) peptide (positions 1177–1178), a lysine–arginine (KR) peptide (positions 1209–1210) and a penta-arginine (RRRRR) sequence (positions 1148–1152). LAR cDNAs containing the various mutations were expressed in murine 300–19 cells and then transfected cells were labelled with [^{35}S]-methionine and LAR proteins were immunoprecipitated and resolved by SDS–PAGE. Changing KK or KR residues at positions 1177–1178 or 1209–1210 to glutamine–leucine (QL) residues, had no effect on LAR processing: both the 150 kDa and 85 kDa LAR subunits were present (Figure 4, lanes 3 and 4). When the penta-arginine sequence (RRRRR) was mutated to RANNN or RARNR, the LAR proteins harboring these mutations were not processed, i.e. precursor proteins with an apparent M_r of $\sim$200 000 were present (Figure 4, lanes 5 and 6). FACS analysis showed that these two mutant LAR proteins were, however, expressed on the cell surface as efficiently as wild-type LAR (data not shown). This result suggested that one or two of the arginines (amino acid positions 1149 and 1151) within the LAR penta-arginine sequence are essential for LAR processing. To delineate further the essential amino acid(s) within the penta-arginine sequence necessary for LAR processing, two mutant LARs containing single amino acid substitutions at amino acid positions 1149 or 1151 were constructed. The LAR containing the RRRRR → RARRR

Tissue	Tested Positive Per Total	Intensity of Staining (0-3)
Adrenal:		
Cortex	3/3*	2
Medulla	0/3	0
Bladder:	0/3	0
Brain:		
Glia	0/3	0
Neurones	0/3	0
Meninges	0/3	0
Breast:		
Acini	3/3**	3
Ducts	3/3**	3
Cervix, uterine:	0/3	0
Colon:	0/3	0
Esophagus:		
Epithelium	3/3***	2
Heart:	4/4°	1-2
Kidney:		
Glomeruli	3/3***	2
Tubules	3/3°°	2
Liver:		
Hepatocytes	0/3	0
Bile ducts	0/3	0
Kuppfer cells	0/3	0
Lung:		
Bronchial cells	2/2***	1-2
Alveolar cells	0/3	0
Pheumocytes	0/3	0
Muscle, Skeletal:	0/3	0
Nerve, Peripheral:		
Fibroblasts	3/3	1-2
Neural axons	0/3	0
Schwann cells	0/3	0
Ovary:	0/3	0
Pancreas:		
Endocrine cells	0/3	0
Exocrine cells	3/3°°	2
Ducts	3/3°°	2
Placenta:	1/1°°	2
Skin:		
Epidermis	3/3***	1
Adnexa	0/3	0
Small Inestine:		
Crypt epithelium	0/3	0
Luminal epithelium	0/3	0
Spinal Cord:	0/2	0
Spleen:		
Red pulp	0/3	0
White pulp	0/3	0
Stomach:	0/3	0
Testis:		
Germinal cells	3/3	1
Peritubular myoid cells	3/3	2
Thyroid:	3/3°°	2
Tonsils:		
Lymphoid cells	0/3	0
Epithelium	3/3***	1-2
Trachea:		
Epithelium	1/1***	2
Glands	3/3***	2
Uterus:		
Endometrium	2/2°°	1
Myometrium	3/3	2

* in region of plasma membrane
** myo-epithelial cells
*** basement membrane and epithelium
° plasma membrane of myocytes
°° basement membrane

Fig. 6. LAR tissue expression. The indicated tissues were immunostained as described in Materials and methods using the anti-LAR 11.1A mAb. This figure does not include the positive vascular and bowel smooth muscle and fibroblast staining seen in various tissues. The intensity of the staining was scored using an arbitrary scale as follows: 0, no staining; 1, weak staining; 2, moderate staining; and 3, strong staining.

(R1149A) mutation was not proteolytically processed (Figure 4, lane 7), whereas the RRRRR → RRRAR (R1151A) mutation was proteolytically processed into 150 kDa and 85 kDa proteins (Figure 4, lane 8). Therefore, a single amino acid substitution (R1149A) inhibits processing of the LAR precursor protein. This result provides strong evidence that Arg1149 is a part of the endoprotease recognition/cleavage site (see Discussion). Furthermore, proteolytic cleavage of the LAR proprotein does not appear to be essential for LAR surface expression, because unprocessed LAR could also be expressed. The 300-19 cells that expressed uncleavable mutant LAR were not morphologically different and did not have different growth rates from the parental cells; uncleaved LAR had comparable PTPase activity with that of wild-type LAR (data not shown).

The LAR 150 kDa E-subunit is shed during cell growth

HeLa cells grown to a high cell density (3×10^5 cells/cm^2) expressed ~3-fold less cell surface LAR than HeLa cultures grown to a lower density (4×10^4 cells/cm^2; Figure 5A). Decreased cell surface expression of LAR was specific, because the same cells expressed identical amounts of HLA class I antigen at either cell density (Figure 5A). To determine if the decreased LAR surface density could result from receptor shedding, HeLa cells were metabolically labelled with [^{35}S]methionine for a period of 15 min and then chased with medium containing excess non-radioactive methionine for 0, 1 or 6 h. Cell extracts and supernatants were separately harvested at the different time points and immunoprecipitations using anti-LAR or control mAbs were carried out. Consistent with the results of Figure 2C, only LAR precursor protein (~200 kDa) was present in cell extracts at time 0 h, while essentially all precursor was processed to the 150 kDa and 85 kDa subunits by 1 h; at 6 h, all precursor LAR protein was processed (Figure 5B, lane 7). As seen in Figure 5B, immunoprecipitations from the supernatants of pulse-labelled confluent HeLa cells (~1.5×10^5 cells/cm^2) revealed that following the 6 h chase, the 150 kDa E-subunit, but not the 85 kDa P-subunit, was present in the supernatant (lane 5). The 150 kDa proteins that were immunoprecipitated from cell extracts or cell culture supernatant had exactly the same M_r as determined by SDS–PAGE analysis (Figure 5B, lanes 5 and 7). Based on densitometric analysis of appropriate autoradiograms shown in Figure 5B, we estimate that 3% of the pulse labelled LAR E-subunit was shed during the 6 h chase period. To confirm that the 150 kDa protein shed from HeLa cells was LAR E-subunit, immunoprecipitation studies using supernatants of 300–19 cell lines expressing deletion mutants of LAR were performed. The supernatant of the 300-19 (LAR) cells also contained a 150 kDa protein but no 85 kDa protein, while the 300–19 (LAR Δ503–715) cells shed a protein of the expected M_r of 125 000. Furthermore, there was no material reactive with anti-LAR mAb in the supernatants of cells expressing the non-cleavable LAR Δ1125–1198 mutant, even after an extended labelling period (data not shown). Therefore, the LAR E-subunit is specifically shed from the cell surface. It remains to be determined, however, whether the LAR receptor shedding alone accounts for the down-regulation of LAR expression on high density cultures of HeLa cells. Other mechanisms, such as specific inhibition of LAR protein synthesis, could also be involved. Treatment of HeLa cells with phorbol myristate acetate (PMA), concanavalin A, human epidermal growth factor or human transforming growth factor-β did not alter significantly the amount of shed LAR E-subunit as determined by FACS analysis (data not shown).

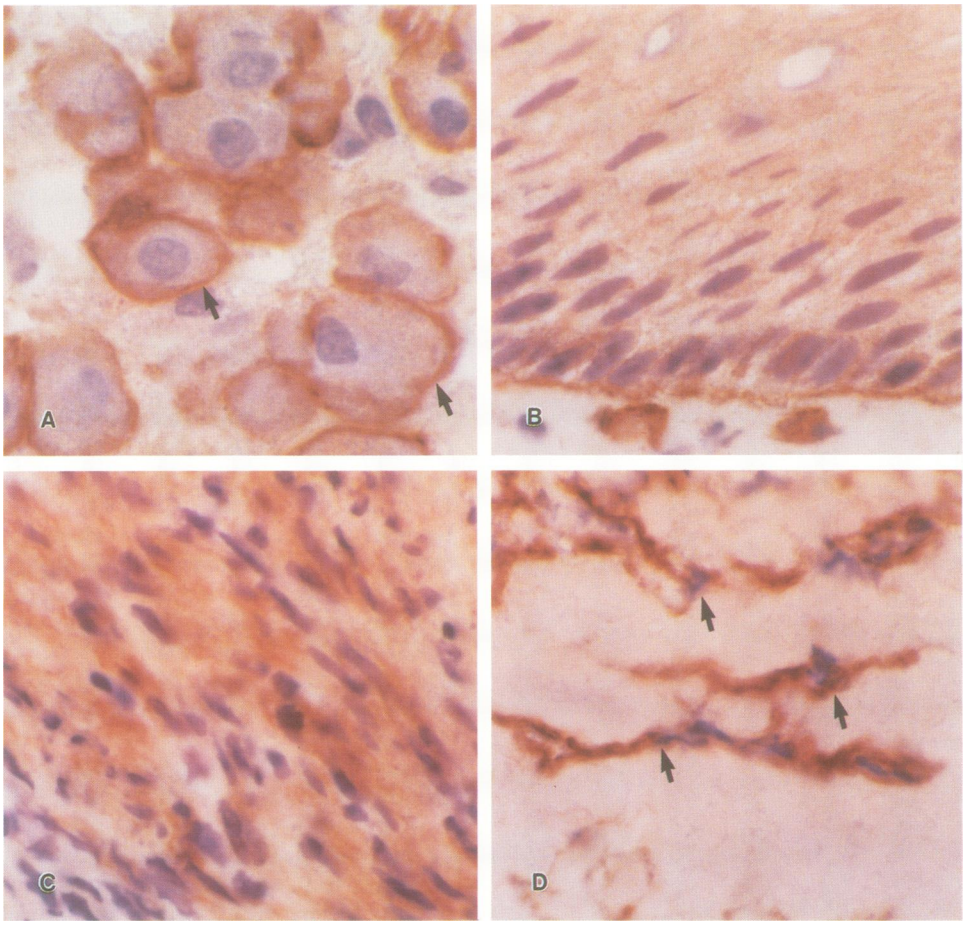


Fig. 7. Immunohistochemical staining of human tissues using anti-LAR mAb. Immunohistochemistry was done as described in Materials and methods using the anti-LAR mAb 11.1A and an avidin–biotin–peroxidase technique with the brown chromogen diaminobenzidine and with hematoxylin counterstain. Final magnification ×1400. (**A**) Expression of LAR by adrenal cortical epithelial cells, notably in the region of the plasma membrane, e.g. where indicated by arrows. (**B**) Expression of LAR by esophageal epithelium with staining also seen over basement membrane. (**C**) Diffuse LAR expression on myometrium (smooth muscle cells). (**D**) Expression of LAR in the adrenal capsule by cells with the morphology of fibroblasts, e.g. where indicated by arrows.

LAR has a broad tissue distribution

The tissues stained with the anti-LAR 11.1A mAb and the intensity of staining are listed in Figure 6. There was expression of LAR on epithelia in various sites including skin, esophagus, respiratory mucosa, glomeruli and tonsils (Figure 7A and B). This staining was usually diffusely present over these epithelia, and was associated with staining in the region of the basement membrane (basement membrane staining was also seen using the anti-LAR mAb 38.1B). The antibody binding was often clearly over basement membrane itself, rather than just over the basal portion of epithelial cells. In addition, smooth muscle and cardiac muscle, but not skeletal muscle, were seen to express LAR. The smooth muscle staining included myometrium (Figure 7C), bowel wall and vascular smooth muscle, which stained strongly. Cells with a morphology similar to fibroblasts stained in various tissues (Figure 7D), notably within the peripheral nerve, as did pericytes. Endothelial cells bound 11.1A in some of the tissues examined, such as spleen, small intestine and spinal cord. Hematopoietic cell expression of LAR was not identified.

The LAR gene is located on chromosome 1

The *LAR* gene was mapped on human metaphase chromosomes by *in situ* hybridization (Marth *et al.*, 1986) using a ^{3}H-labelled LAR18 cDNA probe (Streuli *et al.*, 1988a); a total of 53 metaphase cells were examined. Of the 85 hybridization sites scored, 12 (14% of the sites) were located between bands p32 and p34 of the short arm of chromosome 1 (Figure 8). The largest number of grains was at bands p32 and p33. There was no significant hybridization to other chromosomes.

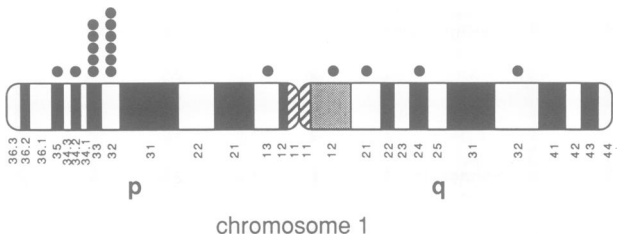


Fig. 8. Regional localization of the *LAR* gene to human chromosome 1 band p32–33. The distribution of autoradiographic grains on a diagram of human chromosome 1 is shown. There was no significant hybridization to other chromosomes.

Discussion

We have shown using immunohistochemistry that the LAR receptor-linked PTPase is expressed *in vivo* on various cells,

including epithelial cells, smooth muscle cells, fibroblasts and cardiac myocytes. Furthermore, we have shown that LAR is cleaved intracellularly into two subunits that are then non-covalently associated on the cell surface and that the extracellular E-subunit can be released from the cell surface. The finding that a tertiary culture of normal human epidermal keratinocytes expressed a heterodimeric LAR structure strongly suggests that *in vivo* LAR is also expressed as a complex of two subunits. The LAR 150 kDa E-subunit contains the CAM-like region including the Ig and FN-III domains, whereas the 85 kDa P-subunit contains a short segment of the extracellular region, the transmembrane peptide and the cytoplasmic PTPase domains (summarized in Figure 3F). Recently, it has been suggested that the neuron−glia CAM (Ng−CAM) is also composed of two, non-covalently associated subunits that are derived from a proprotein (Burgoon *et al.*, 1991). Processing of the LAR proprotein was inhibited by the single amino acid substitution R1149A. Paired dibasic sequence motifs are the endoprotease recognition/cleavage sites of a large number of proproteins (Barr, 1991). Therefore, it is probable that the endoprotease recognition sequence of LAR includes Arg1149, and that cleavage of the LAR proprotein occurs following Arg1152. The human HPTPδ PTPase contains a tetra-basic sequence (RKRR) in the analogous position to the LAR penta-arginine sequence, suggesting the possibility that HPTPδ may also be processed and expressed as a two subunit structure. Cleavage of the insulin receptor proprotein occurs following the sequence RKRR (Ullrich *et al.*, 1985) and mutations at the P4, but not the P2, position have been shown to be necessary for insulin proreceptor processing (Yoshimasa *et al.*, 1990; residues within a four residue motif are labelled as P4.P3.P2.P1, reading left to right). Similarly, mutations at the P4 position (R1149), but not at the P2 position (R1151), of LAR are critical for cleavage of the LAR proprotein. Therefore, the endoprotease responsible for cleaving the LAR proprotein may be one of the subtilisin-like endoproteases, which have been shown to recognize paired dibasic motifs (Wise *et al.*, 1990; Bresnahan *et al.*, 1990; Thomas *et al.*, 1991; Benjannet *et al.*, 1991).

What is the functional significance of the two subunit LAR structure? Clearly, proteolytic processing is not required for surface expression, because uncleaved mutant LAR proteins (e.g. LAR R1149A) can be expressed on the cell surface. Proteolytic processing might be essential for a functional conformation of the LAR molecule. For example, the two subunit structure may be necessary to transmit the receptor-occupancy signal following ligand binding. Cleavage of insulin receptor is necessary for high affinity insulin binding as well as efficient signal transduction following ligand binding, but not for receptor expression (Williams *et al.*, 1990). Indeed, naturally occurring insulin proreceptor processing mutations at the P1 position may cause insulin-resistant diabetes mellitus (Kobayashi *et al.*, 1988; Yoshimasa *et al.*, 1988).

Unlike the α and β subunits of the insulin receptor, however, the LAR E- and P-subunits are not covalently linked, allowing the E-subunit to be shed from the cell surface. Shedding of the extracellular region of several receptors, including the colony stimulating factor 1 receptor, tumor necrosis factor receptor and leukocyte adhesion molecule-1, has been observed (Downing *et al.*, 1989; Porteu and Nathan, 1990; Spertini *et al.*, 1991). Shedding of these receptors occurs through activation of cell surface-associated protease(s) that specifically cleave the receptors following cell activation. Shedding seems to reduce the responsiveness of the cells to the cognate ligands. Similarly, the shedding of LAR might be a way to regulate the sensitivity of cells to LAR−ligand interactions. Mechanistically, however, the shedding of the LAR E-subunit seems distinct from the shedding of the other receptors. LAR is expressed on the cell surface as a two-subunit structure and shedding may occur by a conformational alteration that reduces the affinity between the E- and P-subunits. Alternatively, subunit dissociation induced by ligand binding or some other mechanism may alter the LAR PTPase activity. Artificial dissociation of the receptor domain from the cytoplasmic domain by proteolytic cleavage activates insulin receptor PTK activity and LCA PTPase activity (Leef and Larner, 1987; Shoelson *et al.*, 1988; Tonks *et al.*, 1990). The *in vivo* staining of basement membrane using two distinct anti-LAR mAb (both of which recognize the LAR E-subunit) may be due to shed LAR E-subunit bound to the basement membrane. However, the significance of this phenomenon is presently unclear. Finally, shed LAR E-subunit might also have a cytokine-like or 'decoy' function, thereby competing with cell surface LAR for ligand binding.

The *LAR* gene was located by *in situ* hybridization to chromosome 1 at band p32 or p33. Several candidate tumor suppressor genes have been mapped within or close to this region. Loss of heterozygosity has frequently been observed in the 1p32−35 region in patients with phaeochromocytoma and medullary thyroid carcinoma (Mathew *et al.*, 1987; Samaan *et al.*, 1989). More distal to this region, a candidate neuroblastoma suppressor gene (Fong *et al.*, 1989), and a candidate ductal breast carcinoma suppressor gene (Genuardi *et al.*, 1989) have been mapped. Although it remains to be determined whether *LAR* gene deletion or mutation is associated with these or any other malignancies, the possibility that PTPases may be tumor suppressor genes is supported by the mapping of the *PTPγ* gene to a region of chromosome 3 which is frequently deleted in renal cell carcinomas and lung carcinomas (LaForgia *et al.*, 1991).

Materials and methods

Plasmid constructions

pMT.LAR and pMT.LCA.1 were constructed by inserting the full-length LAR cDNA sequence (Streuli *et al.*, 1988a) or full-length human LCA.1 cDNA (Streuli *et al.*, 1987) into the unique *Eco*RI site of the eukaryotic expression vector, pMT2 (Bonthron *et al.*, 1986). pMT.LAR−LCA was derived from pMT.LCA and pMT.LAR by ligating appropriate restriction fragments. The resulting hybrid LAR−LCA protein contains, in the following order, the LCA leader sequence, LCA amino acids 1−8 and 202−218, LAR amino acids 1−334 and LCA amino acids 214−1281 (numbering of LCA and LAR amino acid residues according to Streuli *et al.* (1987 and 1988a, respectively). Several LAR deletion mutants were generated by combining appropriate restriction fragments, while other deletion mutants and single or multiple amino acid substitution mutants were generated by oligonucleotide-directed mutagenesis essentially according to Foss and McClain (1987). The amino acid residues deleted in the various pMT.LAR constructs are given in Figure 3B, while the amino acid residues substituted are given in the Figure 4 legend. Structures of plasmid constructs were confirmed by restriction mapping and/or by nucleotide sequencing using the method of Sanger *et al.* (1977) using T7 DNA polymerase (Tabor and Richardson, 1989).

Transfections

The murine pre-B lymphocyte line 300-19 transformed with the Abelson virus (Alt *et al.*, 1984) was grown in medium A (RPMI medium (Gibco,

Grand Island, NY), 10% FCS, 0.05 mM 2-mercaptoethanol, 50 μg/ml gentamicin sulfate and 2 mM L-glutamine). Cells were transfected by electroporation (Chu *et al.*, 1987) with plasmid DNA using the Cell Porator Electroporation System (Bethesda Research Laboratories) essentially as described by the manufacturer. pMT.LAR constructs and pSV2neoSP (Streuli and Saito, 1989) were linearized before transfections with *Sspl* and *Pvul*, respectively. The parameters for electroporation were ~50 μg pMT.LAR DNA and ~3 μg pSV2neoSP DNA/5 × 10^6 cells/0.5 ml DMEM (Gibco) at 0°C, 1.6 mF, 250 V/0.4 cm, using the high Ohm setting. Transfected cells were selected in medium A supplemented with 2 mg/ml Geneticin (Gibco). Geneticin-resistant clones were maintained in medium A supplemented with 0.5 mg/ml Geneticin.

Monoclonal antibodies

To generate high affinity anti-LAR mAbs that recognize the extracellular region of LAR, Balb/c mice were immunized with a murine B lymphoma cell line expressing human LAR [300-19 (LAR)]. Because the parental 300-19 cells are essentially congeneric with the mice immunized, the human LAR protein is a dominant antigen. Initially, a LAR−LCA hybrid cDNA was constructed that contained the three LAR Ig domains fused to the 180 kDa LCA isoform (Figure 1). This hybrid cDNA, cloned into the pMT-2 expression plasmid, was co-transfected with pSV2neoSP into 300-19 cells and the expression of the hybrid molecule on resulting cell lines [300-19 (LAR−LCA)] was monitored using the available anti-LCA mAbs, UCHL-1 and GAP8.3. UCHL-1 binds to the N-terminal region of the 180 kDa LCA isoform, while GAP8.3 binds to a more C-terminal region of LCA (Streuli *et al.*, 1988b). Hybridoma supernatants were screened for binding to 300-19 (LAR−LCA) cells and lack of binding to a transfected 300-19 cell line that expressed only human LCA [300-19 (LCA)]. An anti-LAR mAb 108.4A was thus identified and used to identify a murine B cell transfectant cell line, 300-19 (LAR), that expressed LAR protein. 300-19 (LAR) cells were then used as an immunogen as described above. The supernatants from the resulting hybridomas were screened for binding to 300-19 (LAR), 300-19 (LAR−LCA) or 300-19 (LCA) cells. mAbs that bound to both the LAR−LCA hybrid and LAR were specific to the LAR Ig domains (38.1B, 75.3A and 108.4A), while antibodies that bound only to LAR were specific to the LAR FN III domains (11.1A, 71.2E, 128.4A and 136.1; summarized in Figure 1). None of these anti-LAR mAbs reacted with purified intracellular LAR catalytic domain protein. 300-19 (LAR−LCA) or 300-19 (LAR) cells were intraperitoneally injected into Balb/c mice at 3 week intervals for a 9−12 week period at 5−10 × 10^7 cells/injection. Splenocytes from immunized mice were fused to NS-1 myeloma cells and HAT-resistant hybridomas were selected as previously described (Kohler and Milstein, 1975). Anti-LAR mAbs and isotype matched (IgG1) control mAbs, were purified using protein G−Sepharose beads (GammaBind Plus, Genex Corp., Gaithersburg, MD) as described by the manufacturer. The anti-LCA mAbs UCHL-1 (CD45RO) and anti-2H4 (CD45RA), and the anti-HLA class I heavy chain mAb, W6/32, have been described previously (Streuli *et al.*, 1988b; Barnstable *et al.*, 1978).

Cells, cell labelling and immunoprecipitations

The epitheloid carcinoma cell line HeLa (ATCC CCL 2) and the epidermoid carcinoma cell line A-431 (ATCC CRL 1555), were grown in DMEM (Gibco) supplemented with 10% FCS, 50 μg/ml gentamicin sulfate and 2 mM L-glutamine. Normal human epidermal keratinocyte (NHEK) cells were obtained from Clonetics Corp. (San Diego, CA) and tertiary cultures were established as recommended by the supplier. Cells were surface-labelled with ^{125}I by the lactoperoxidase method (Philips and Morrison, 1971). Briefly, cells were washed in PBS, resuspended at ~2 × 10^7 cells in 0.2 ml PBS, 1 mCi carrier-free ^{125}I (sodium salt; Amersham) and 1 U lactoperoxidase (Sigma) were added, then 10 μl 0.037% H_2O_2 was added three times at 2 min intervals. After labelling, cells were washed in PBS, lysed in NP-40 lysis buffer [1% Nonidet P-40, 150 mM NaCl, 50 mM Tris−HCl (pH 8.0), 5 mM EDTA, containing 1 mM PMSF] and lysates were centrifuged in a microfuge to remove insoluble material. Alternatively, cells were metabolically labelled with [^{35}S]methionine (~1100 mCi/mmol) (New England Nuclear, Boston, MA) in DMEM without L-methionine (Gibco) supplemented with 1% (v/v) dialyzed FCS, 20 mM HEPES (pH 7.2) for 4−6 h (unless indicated otherwise) at 37°C (~5 × 10^6 cells/0.5 mCi [^{35}S]methionine/ml). Cell culture supernatants were harvested at the end of the labelling period and adjusted to 1 mM PMSF. After labelling, cells were washed in PBS and lysed by NP-40 lysis buffer as described above. Cell lysates (or cell culture supernatants) were then precleared two to three times with ~30 μg purified, isotype-matched mAb and 25 μl protein G−Sepharose beads for 8−14 h. For immunoprecipitations, ~30 μg anti-LAR mAb (1:1:1 mixture of 75.3A, 11.1A and 128.4A; unless noted otherwise) or ~30 μg control isotype-matched mAb and 25 μl protein G−Sepharose beads were added per ml precleared lysate for 8−14 h. Immunoprecipitates were then washed five times with NP-40 lysis buffer and analyzed by SDS−PAGE (7.5% or 10% gels) using reducing conditions (unless noted otherwise) followed by autordiography (12−72 h, unless noted otherwise).

Phosphatase assay

Phosphatase assays were done as described by Streuli *et al.* (1990) using [^{32}P]Tyr-Raytide substrate and LAR PTPase immunoprecipitated from HeLa or 300-19 (LAR) cells as described above, except that cells were not radiolabelled prior to immunoprecipitations.

Flow cytometry

Flow cytometric analysis of cell lines was performed by means of indirect immunofluorescence with fluorescein-conjugated goat anti-mouse $F(ab')_2$ on an Epics V cell sorter (Coulter Electronics, Hialeah, FL). For FACS analysis, HeLa cells were washed with PBS, incubated for 30 min at 37°C in PBS containing 0.5 mM EDTA and 0.02% (w/v) NaN_3. Cells were then harvested, purified using a Ficoll gradient and 1 × 10^6 cells were used per staining.

Immunohistochemistry

Blocks of human tissue were collected from surgical or autopsy specimens at the Brigham and Women's Hospital (Boston, MA) and snap frozen in liquid N_2. The latter were used only if they could be frozen within 24 h of death: systematic study has indicated that antigens are well preserved during this post-mortem interval (Knudsen and Pallesen, 1986). The tissues that were analyzed are listed in Figure 6. Cryostat sections (6 μm) were cut and fixed in 2% paraformaldehyde. They were then stained immunohistochemically using primary antibodies and an avidin−biotin−peroxidase technique with the chromogen diaminobenzidine as previously described (Rice *et al.*, 1991). The primary antibodies were anti-LAR 11.1A (IgG1) and in selected instances 38.1B (IgG1). Control non-specific mouse IgG1 (Coulter Immunology) were also used on separate sections at equal concentration; these did not produce staining. Intensity of staining was scored using an arbitrary scale as follows: 0, no staining; 1, weak staining; 2, moderate staining; and 3, strong staining.

Chromosomal location

The LAR18 cDNA clone (Streuli *et al.*, 1988a) was labelled by nick-translation using [^3H]nucleotides to a specific activity of 4 × 10^7 c.p.m./μg. *In situ* hybridization to metaphase chromosomes from lymphocytes of a normal male donor was carried out using the LAR18 probe at a concentration of 0.2 ng/μl hybridization mixture as described (Marth *et al.*, 1986). The slides were exposed for five days. The chromosomes were identified by Q-banding (Marth *et al.*, 1986).

Acknowledgements

We thank Drs Paul Anderson, Charles C.Richardson and Michael Robertson for critical review and valuable discussion, Dr Tim Ernst for synthesizing oligonucleotides, Dr Chikao Morimoto for advice on hybridoma production, Dr Mary Sunday for initial help with the immunohistochemistry and Dr Stuart F.Schlossman for encouragement and support. This work was supported by National Institutes of Health grants CA51132 and AI26598 to H.S. and AG01751 and March of Dimes Grant 1-1019 to C.M.D. M.S. was supported by a Cancer Research Institute/Jesselson Foundation Fellowship.

References

Alt,F.W., Yancopoulos,G.D., Blackwell,T.K., Wood,C., Thomas,E., Boss,M., Coffman,R., Rosenberg,N., Tonegawa,S. and Baltimore,D. (1984) *EMBO J.*, **3**, 1209−1219.

Barnstable,C.J., Bodmer,W.F., Grown,G., Galfare,G., Milstein,C., Williams,A.F. and Ziegler,Z. (1978) *Cell*, **14**, 9−23.

Barr,P.J. (1991) *Cell*, **66**, 1−3.

Benjannet,S., Rondeau,N., Day,R, Chretien,M. and Seidah,N.G. (1991) *Proc. Natl. Acad. Sci. USA*, **88**, 3564−3568.

Bishop,M. (1991) *Cell*, **64**, 235−248.

Bonthron,D.T. *et al.* (1986) *Nature*, **324**, 270−273.

Bresnahan,P.A., Leduc,R., Thomas,L., Thorner,J., Gibson,H.L., Brake,A.J., Barr,P.J. and Thomas,G. (1990) *J. Cell Biol.*, **111**, 2851−2859.

Brown-Shimer,S., Johnson,K.A., Lawrence,J.B., Johnson,C., Bruskin,A.,

Green,N.R. and Hill,D.E. (1990) *Proc. Natl. Acad. Sci. USA*, **87**, 5148–5152.
Burgoon,M.P., Grumet,M., Mauro,V., Edelman,G.M. and Cunningham,B.A. (1991) *J. Cell Biol.*, **112**, 1017–1029.
Charbonneau,H., Tonks,N.K., Walsh,K.A. and Fischer,E.H. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 7182–7186.
Chernoff,J., Schievella,A.R., Jost,C.A., Erikson,R.L. and Neel,B.G. (1990) *Proc. Natl. Acad. Sci. USA*, **87**, 2735–2739.
Chu,G. Hayakawa,H. and Berg,P. (1987) *Nucleic Acids Res.*, **15**, 1311–1326.
Cool,D.E., Tonks,N.K., Charbonneau,H., Walsh,K.A., Fischer,E.H. and Krebs,E.G. (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 5257–5261.
Downing,J.R., Roussel,M.F. and Sherr,C.J. (1989) *Mol. Cell. Biol.*, **9**, 2890–2896.
Fearon,E.R. *et al.* (1990) *Science*, **247**, 49–56.
Fischer,E.H., Charbonneau,H. and Tonks,N.K. (1991) *Science*, **253**, 401–406.
Fong,C., Dracopoli,N.C., White,P.S., Merrill,P.T., Griffith,R.C., Housman,D.E. and Brodeur,G.M. (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 3753–3757.
Foss,K. and McClain,W.H. (1987) *Gene*, **59**, 285–290.
Genuardi,M., Tsihira,H., Anderson,D.E. and Saunders,G.F. (1989) *Am. J. Hum. Genet.*, **45**, 73–82.
Gu,M., York,J.D., Warshawsky,I. and Majerus,P.W. (1991) *Proc. Natl. Acad. Sci. USA*, **88**, 5867–5871.
Guan,K., Haun,R.S., Watson,S.J., Geahlen,R.L. and Dixon,J.E. (1990) *Proc. Natl. Acad. Sci. USA*, **87**, 1501–1505.
Hunter,T. (1989) *Cell*, **58**, 1013–1016.
Kaplan,R., Morse,B., Huebner,K., Croce,C., Howk,R., Ravera,M., Ricca,G., Jaye,M. and Schlessinger,J. (1990) *Proc. Natl. Acad. Sci. USA*, **87**, 7000–7004.
Knudsen,L.M. and Pallesen,G. (1986) *Histopathology*, **10**, 1007–1014.
Kobayashi,M. *et al.* (1988) *Biochem. Biophy. Res. Commun.*, **153**, 657–663.
Kohler,G. and Milstein,C. (1975) *Nature*, **256**, 495–497.
Krueger,N.X., Streuli,M. and Saito,H. (1990) *EMBO J.*, **9**, 3241–3252.
LaForgia,S. *et al.* (1991) *Proc. Natl. Acad. Sci. USA*, **88**, 5036–5040.
Leef,J.W. and Larner,J. (1987) *J. Biol. Chem.*, **262**, 14837–14842.
Lombroso,P.J., Murdoch,G. and Lerner,M. (1991) *Proc. Natl. Acad. Sci. USA*, **88**, 7242–7246.
Marth,J.D., Disteche,C., Paravtcheva,D., Ruddle,F., Krebs,E.G. and Permutter,R. (1986) *Proc. Natl. Acad. Sci. USA*, **83**, 7400–7404.
Mathew,C.G.P., Smith,B.A., Thrope,K., Wong,., Royle,N.J., Jeffreys,A.J. and Podner,B.A.J. (1987) *Nature*, **328**, 524–526.
Matthews,J., Cahir,E.D. and Thomas,M.L. (1990) *Proc. Natl. Acad. Sci. USA*, **87**, 4444–4448.
Patthy,L. (1990) *Cell*, **61**, 13–14.
Philips,D.R. and Morrison,M. (1971) *Biochemistry*, **10**, 1766–1771.
Porteu,F. and Nathan,C. (1990) *J. Exp. Med.*, **172**, 599–607.
Rice,G.E., Munro,J.M., Corless,C. and Bevilacqua,M.P. (1991) *Am. J. Pathol.*, **138**, 385–393.
Saito,H. and Streuli,M. (1991) *Cell Growth Differ.*, **2**, 59–65.
Samaan,N.A., Yang,K.-P., Schultz,P. and Hickey,R.C. (1989) *Henry Ford Hosp. Med. J.*, **37**, 132–137.
Sanger,.F, Nicklen,S. and Coulson,A.R. (1977) *Proc. Natl. Acad. Sci. USA*, **74**, 5463–5467.
Shen,S.-H., Bastien,L., Posner,B.I. and Chretien,P. (1991) *Nature*, **352**, 736–739.
Shoelson,S.E., White,M.F. and Kahn,C.R. (1988) *J. Biol. Chem.*, **263**, 4852–4860.
Spertini,O., Freedman,A.S., Belvin,M., Penta,A.C., Griffin,J.D. and Tedder,T.F. (1991) *Leukemia*, **5**, 300–308.
Streuli,M., Hall,L.R., Saga,Y., Schlossman,S.F. and Saito,H. (1987) *J. Exp. Med.*, **166**, 1548–1566.
Streuli,M., Krueger,N.X., Hall,L.R., Schlossman,S.F. and Saito,H. (1988a) *J. Exp. Med.*, **168**, 1523–1530.
Streuli,M., Morimoto,C., Schrieber,M., Schlossman,S.F. and Saito,H. (1988b) *J. Immunol.*, **141**, 3910–3914.
Streuli,M., Krueger,N.X., Tsai,A.Y.M. and Saito,H. (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 8698–8702.
Streuli,M. and Saito,H. (1989) *EMBO J.*, **8**, 787–796.
Streuli,M., Krueger,N.X., Thai,T., Tang,M. and Saito,H. (1990) *EMBO J.*, **9**, 2399–2407.
Tabor,S. and Richardson,C.C. (1989) *J. Biol. Chem.*, **264**, 6447–6458.
Thomas,L., Leduc,R., Thorne,B.A., Smeekens,S.P., Steiner,D.F. and Thomas,G. (1991) *Proc. Natl. Acad. Sci. USA*, **88**, 5297–5301.
Thomas,M.L. (1989) *Annu. Rev. Immunol.*, **7**, 339–369.
Tonks,N.K., Diltz,C.D. and Fischer,E.H. (1990) *J. Biol. Chem.*, **265**, 10674–10680.
Ullrich,A. *et al.* (1985) *Nature*, **313**, 756–761.
Williams,A.F. and Barclay,A.N. (1988) *Annu. Rev. Immunol.*, **6**, 381–405.
Williams,J.F., McClain,D.A., Dull,T.J., Ullrich,A. and Olefsky,J.M. (1990) *J. Biol. Chem.*, **265**, 8463–8469.
Wise,R.J., Barr,P.J., Wong,P.A., Kiefer,M.C., Brake,A.J. and Kaufman,R.J. (1990) *Proc. Natl. Acad. Sci. USA*, **87**, 9378–9382.
Yang,Q. and Tonks,N.K. (1991) *Proc. Natl. Acad. Sci. USA*, **88**, 5949–5953.
Yoshimasa,Y., Seino,S., Whittaker,J., Kakehi,T., Kosaki,A., Kuzuya,H., Imura,H., Bell,G.I. and Steiner,D.F. (1988) *Science*, **240**, 784–787.
Yoshimasa,Y., Paul,J.I., Whittaker,J. and Steiner,D.F. (1990) *J. Biol. Chem.*, **265**, 17230–17237.

Received on September 19, 1991; revised on November 18, 1991