Cell Motility and the Cytoskeleton 14:449–454 (1989)

Views and Reviews

Tyrosine Protein Kinase Substrate p36: A Member of the Annexin Family of Ca^{2+}/Phospholipid-Binding Proteins

Volker Gerke

Max-Planck-Institute for Biophysical Chemistry, Department of Biochemistry, Göttingen, FRG

INTRODUCTION

Ten years ago a protein of apparent M_r 36,000 was identified as a major cellular target for the transforming tyrosine kinase encoded by the src oncogene. In a series of in vivo labeling experiments, Radke and Martin [1979], Radke et al. [1980], and Erikson and Erikson [1980] showed that p36 is phosphorylated on tyrosine residues when chicken embryo fibroblasts are transformed by Rous sarcoma virus. This finding sparked much interest in p36 and initiated the search for its cellular function(s). Several studies proposed a role in membrane-cytoskeleton interactions, membrane fusion, or signal transduction. While the exact physiological role of this $pp60^{src}$ substrate is still unclear, intensive research has led to a detailed biochemical and structural characterization of p36, which will be discussed in this review.

BIOCHEMICAL PROPERTIES AND SUBCELLULAR LOCALIZATION

Initially, p36 was identified in chicken embryo fibroblasts [Radke and Martin, 1979; Radke et al., 1980; Erikson and Erikson, 1980] and was isolated from these cells using conventional extraction and chromatographic procedures [Erickson and Erikson, 1980; Erikson et al., 1984]. The purification of milligram amounts of p36 from mammalian intestinal epithelium was established by exploiting the fact that p36 could be specifically extracted from cytoskeletal and/or membrane structures prepared in the presence of Ca^{2+}, once the divalent cation was chelated by EGTA [Gerke and Weber, 1984]. Following either approach, two distinct forms of p36 could be purified—monomeric p36 and p36 complexed with a cellular protein ligand, p11. The complex, known as protein I or calpactin I, consists of two p36 and two p11 chains [Gerke and Weber, 1985a; Glenney, 1986].

Both monomeric p36 and the $p36_2p11_2$ complex show direct Ca^{2+}-binding, albeit with rather low affinity ($K_d \sim 10^{-4}$ M). In the presence of Ca^{2+} both forms of p36 are able to interact with negatively charged phospholipids, preferentially those found enriched on the cytoplasmic face of the plasma membrane [for review see Klee, 1988]. A mutual influence of Ca^{2+}- and phospholipid-binding is indicated by the fact that the affinity for Ca^{2+} is greatly increased in the presence of phosphatidylserine. Under such conditions at least two Ca^{2+}-sites are detected on each p36 chain with a K_d of 4.5 μM [Glenney, 1986]. Monomeric p36 and the $p36_2p11_2$ complex are also able to interact with cytoskeletal elements in vitro. In the presence of Ca^{2+}, they bind to F-actin and non-erythroid-spectrins [Gerke and Weber, 1984, 1985a]. Based on the in vitro binding to *Ca*$^{2+}$, *lip*id, and F-*actin* the name calpactin I was proposed for the $p36_2p11_2$ complex [Glenney, 1986].

While p36 exists within the cell either as a monomer or as the $p36_2p11_2$ heterotetramer, p11 has so far only been found complexed to p36. In vitro, p11 can be separated from p36 following denaturation in 9 M urea or 6 M guanidine-HCl [Gerke and Weber, 1985a; Glenney, 1986]. Renatured p11 is a dimer, and sequence analysis indicates that it belongs to the S-100 family of Ca^{2+}-binding proteins [Gerke and Weber, 1985a,b; Glenney and Tack, 1985]. However, it lacks functional EF-hands [Kretsinger, 1987], which form the Ca^{2+}-binding sites

Accepted July 17, 1989.

Address reprint requests to Volker Gerke, Max-Planck-Institute for Biophysical Chemistry, D-3400 Göttingen, FRG.

in S-100. Consequently, p11 has lost the ability to bind Ca^{2+} and shows a Ca^{2+}-independent interaction with p36 [Gerke and Weber, 1985b; Glenney, 1986].

A battery of polyclonal and monoclonal antibodies have been employed to study the tissue distribution and sub-cellular localization of p36 and p11 [for review, see Klee, 1988]. Both polypeptides are coordinately expressed and are particularly abundant in intestinal epithelium, lung, and placenta but are undetectable or present at very low levels in brain, skeletal muscle, liver, and erythrocytes [Zokas and Glenney, 1987]. Immunofluorescence microscopy revealed a submembraneous localization of p36 and p11 in a variety of cultured cells. Immunocytochemistry in combination with electron microscopy allowed a more precise localization [Semich et al., in press]. Both p36 and p11 are associated with the cytoplasmic face of the membrane showing a dense and very regular distribution even at sub-micromolar Ca^{2+}-concentrations. When cells are treated with detergent in the presence of Ca^{2+} prior to fixation, immunofluorescence microscopy reveals that the $p36_2p11_2$ complex stays in the submembraneous cytoskeleton. A similar treatment leads at least to a partial solubilization of monomeric p36, indicating that the two forms of p36 (monomer and heterotetramer) might exist in different locations within the cell [Zokas and Glenney, 1987]. p11, on the other hand, is restricted to the submembraneous network [Zokas and Glenney, 1987]. Since p11 itself is not able to interact with phospholipids or cytoskeletal structures, its presence in the cortical cytoskeleton suggests an interaction with p36 in vivo. Even when microinjected into living cells, p11 will travel to the cell periphery and stay in the cortical network, presumably owing to its binding to endogenous p36 [Osborn et al., 1988]. While both forms of p36, the monomer and the heterotetramer ($p36_2p11_2$), coexist within the cell, their relative amounts seem to vary in different cell types. Intestinal epithelial cells contain more than 90% of their p36 in the complexed form, while chicken embryo fibroblasts transformed by Rous sarcoma virus may have a 50% excess of monomeric p36 [Gerke and Weber, 1984; Erikson et al., 1984].

The functional significance of varying relative amounts and a different sub-cellular distribution of monomeric p36 and the $p36_2p11_2$ complex is not understood. However, neither form shows the concentration in focal adhesion plaques observed for $pp60^{src}$ [Rohrschneider, 1980; Nigg et al., 1982]. This might explain why only a small portion of p36 (not more than 10%) is tyrosine-phosphorylated in Rous sarcoma virus-transformed cells [Radke et al., 1980]. The portion of p36 that actually becomes phosphorylated might reside in close proximity to $pp60^{src}$, showing a localization different from the overall distribution of p36 and $p36_2p11_2$.

STRUCTURAL CHARACTERISTICS

Elucidation of the primary structure of p36 [Saris et al., 1986; Huang et al., 1986] revealed that it belongs to a newly described multigene family, also known as the annexin or lipocortin family [for review, see Crompton et al., 1988; Klee, 1988; Burgoyne and Geisow, 1989]. Members of this family show a Ca^{2+}-dependent interaction with phospholipids and share a common structural element: a segment of 70 to 80 amino acids, which is tandemly repeated along the polypeptide chain. p36, as all other 35–40 kD members of the family, shows four repeats. The individual repeat motifs exhibit homologies with each other, not only within an individual protein but also with other members of the annexin family. In all annexins, the sum of the repeat motifs, which comprise the so-called core domain, is preceeded by an N-terminal tail, which is variable in sequence and length (see Fig. 1). Limited proteolysis also defines the two regions of the molecules. The N-terminal tail is sensitive to mild proteolytic treatment, whereas the core is remarkably resistant.

A careful examination of the p36 sequence reveals no homologies to sequences, which are proposed to be responsible for Ca^{2+}- or phospholipid-binding in other proteins. Most notably, the classical helix-loop-helix structure (''EF-hand''), which is known to form the Ca^{2+}-binding sites in proteins such as parvalbumin and calmodulin [Kretsinger, 1987], is absent from p36. So far, the only outstanding structural feature described within the p36 chain and all other annexins is a stretch of 17 amino acids that may form a helix-loop structure involved in Ca^{2+}-binding [Geisow et al., 1986]. This so-called ''endonexin-fold'' is present in each repeat motif (see Fig. 1) and shows a remarkable degree of sequence conservation in the different members of the annexin family.

The repetitive structure of the core domain of p36 (as well as other annexins) may reflect the presence of four functionally independent units. In the case of p36, no experimental evidence has emerged yet for this ''1 repeat motif-1 Ca^{2+}/lipid-binding site'' hypothesis. In fact, equilibrium dialysis revealed only two Ca^{2+}-binding sites for p36 in the presence of phospholipid [Glenney, 1986]. Lipocortin I (p35), on the other hand, a different member of the annexin family with approximately 50% sequence identity to p36, contains four Ca^{2+}-binding sites [Schlaepfer and Haigler, 1987]. In addition, a proteolytic fragment of lipocortin I (amino acids 97 to 178), which harbors the intact repeat motif 2 and parts of motifs 1 and 3, displays the Ca^{2+}-dependent inhibition of phospholipase A_2 characteristic for the entire molecule [Huang et al., 1987]. Since this inhibitory activity is most likely based on the binding of lipocortin

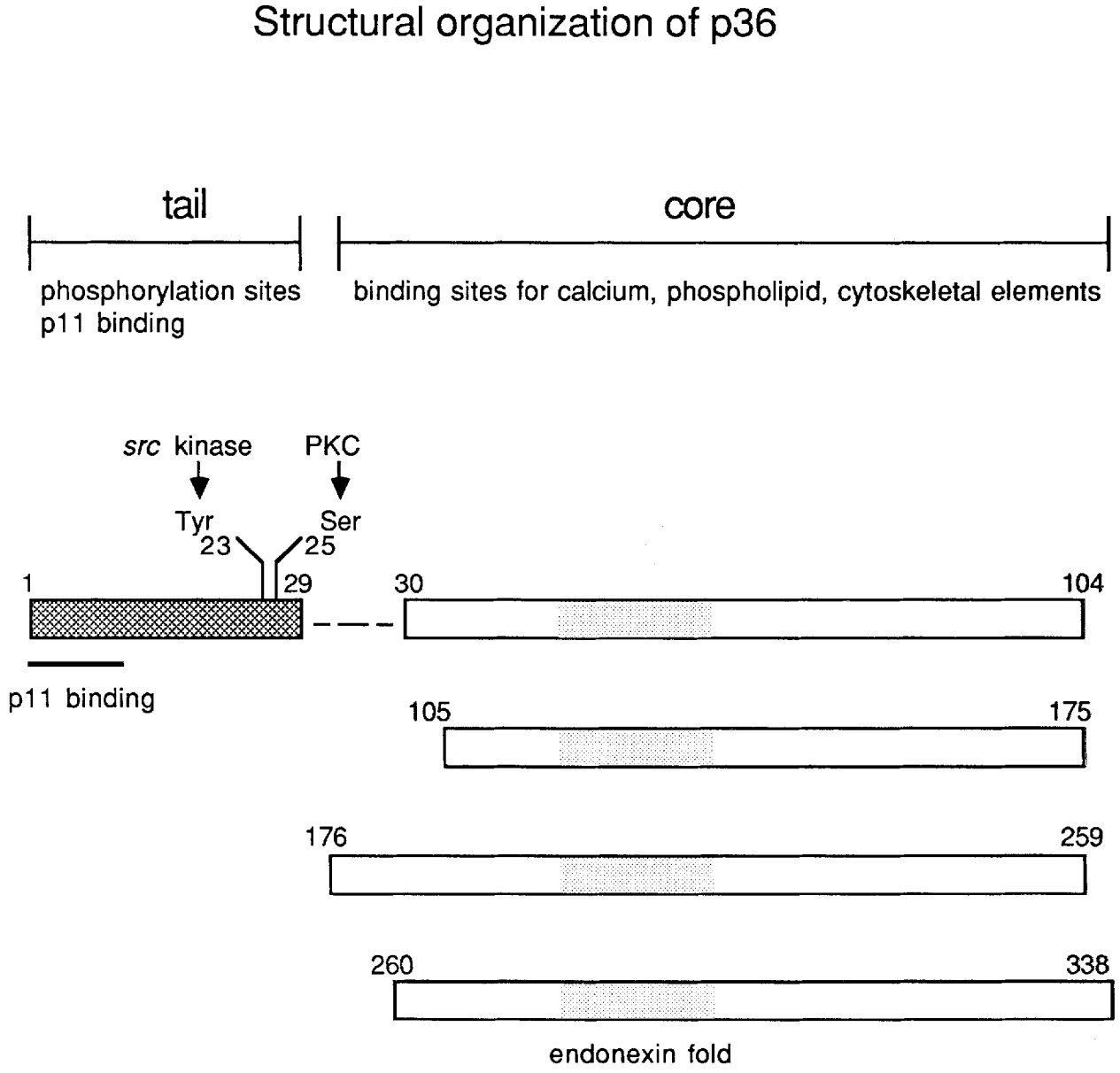

Fig. 1.

I to the phospholipid substrate in the phospholipase A_2 assay [Davidson et al., 1987], a single repeat motif of lipocortin I seems sufficient for the interaction with Ca^{2+} and/or phospholipids.

Sequence parameters in the N-terminal tail of p36 are by far more thoroughly studied than those in the repeat motifs. This relatively short portion of the molecule (29 amino acid residues as defined by a chymotryptic digest, see Fig. 1) harbors the binding site for the p11 subunit as well as the phosphorylation sites of $pp60^{src}$ and a variety of serine/threonine-specific protein kinases. The tyrosine residue phosphorylated in vivo and in vitro by $pp60^{src}$ was mapped to Tyr-23 [Glenney and Tack, 1985]. Ser-25 is a site for protein kinase C in vivo and in vitro [Gould et al., 1986; Johnsson et al., 1986]. Other minor site(s) for kinase C phosphorylation are also present in the p36 tail but have not yet been mapped to a particular serine or threonine residue. In vitro, p36 is also phosphorylated by calmodulin- and c-AMP-dependent protein kinases with the phosphorylation again occurring within the N-terminal 29 residues [Johnsson et al., 1986].

Delineation of the p11 binding site on p36 was greatly facilitated by fluorescence spectroscopy using p36 or synthetic peptides derived from the N-terminal tail, labeled at Cys-8 with the fluorophor prodan [Johns-

son et al., 1988a]. Since p11-binding alters the fluorescence emission of these derivatives, this approach led to the identification of the first 10 residues of the p36 tail as the major p11 binding site. Interestingly, this portion of the molecule is able to form an amphiphatic α-helix. Spatial constraints and steric hindrance are probably responsible for the interference of p11-binding with some phosphorylation events and vice versa. For example, when p36 is phosphorylated by kinase C at certain serine residue(s), it is not able to bind p11 with significant affinity [Johnsson et al., 1986].

Several observations indicate that the N-terminal tail is responsible for the regulation of activities displayed by the protein core. p36 phosphorylated at Tyr-23 by $pp60^{src}$ has an increased Ca^{2+}-requirement for phosphatidylserine binding when compared with the non-phosphorylated form [Powell and Glenney, 1987]. The Ca^{2+}-requirement for the interaction with phospholipids is also changed upon p11 binding to p36. While monomeric p36 needs the presence of 10 μM free Ca^{2+} for 50% binding to phospholipid liposomes, the $p36_2p11_2$ complex shows the same interaction already at 10 nM Ca^{2+} [Powell and Glenney, 1987]. Similarly, the complex is able to induce chromaffin granule aggregation and even fusion of these vesicles at much lower Ca^{2+}-concentrations than monomeric p36. Whereas half-maximal aggregation induced by $p36_2p11_2$ occurs at 1.8 μM Ca^{2+}, only a small amount of aggregation is observed with the p36 monomer even at 1 mM Ca^{2+} [Drust and Creutz, 1988]. Direct evidence for a three-dimensional proximity of the N-terminal tail and the protein core was obtained by mapping the epitope for a monoclonal p36 antibody. Johnsson et al. [1988b] could show that this antibody recognizes a discontinuous epitope, which includes the surroundings of Val-26 in the tail portion of the molecule and Glu-65 in the first repeat motif of the protein core.

POSSIBLE CELLULAR FUNCTION

Biochemical properties and the intracellular location of p36 suggest a structural and functional connection to the plasma membrane. Consequently, it has been proposed that p36 is involved in the membrane-cytoskeletal linkage and/or the control of membrane fusion events in exocytosis [for review, see Burgoyne and Geisow, 1989]. While the latter hypothesis is based on the ability of the $p36_2p11_2$ complex to aggregate and fuse chromaffin granules at micromolar Ca^{2+}-concentrations [Drust and Creutz, 1988], a potential role for p36 in linking membrane and cortical cytoskeleton has been concluded from the binding to phospholipid and non-erythroid spectrin observed in vitro. Despite this interaction and the co-localization of p36 and non-erythroid spectrins at the cytoplasmic side of the plasma membrane, several experiments argue against a simple direct association in vivo. When cells are microinjected with monoclonal antibodies to p36 or p11, an intracellular patching of the $p36_2p11_2$ complex is observed. This leads finally to the formation of large $p36_2p11_2$ aggregates, without affecting the distribution of non-erythroid spectrins [Zokas and Glenney, 1987]. Injection of antibodies to non-erythroid spectrins, on the other hand, leads to aggregates without a disturbing effect on the p36 distribution [Mangeat and Burridge, 1984]. Other concepts for p36 function deal with a potential involvement of p36 and other annexins in the regulation of transmembrane proteins that act as receptors for extracellular components [Burgoyne and Geisow, 1989]. This control could be mediated by a yet to be proven interaction of p36 with the cytoplasmic portion of the receptors.

p36 and other annexins are also discussed as possible "second messengers" in the anti-inflammatory response to steroids. This hypothesis arose as soon as lipocortin I was identified as a "secreted" inhibitor of pancreatic phospholipase A_2 [for review, see Crompton et al., 1988; Klee, 1988]. Although p36 has been found to display the same activity in vitro, it is yet unclear whether it would regulate intracellular phospholipases in vivo. Indeed, it has been shown recently that the inhibitory effect is most likely due to binding of p36 to the phospholipid substrate used in the assay and is not based on a specific interaction with the enzyme [Davidson et al., 1987]. In addition, no induction of p36 expression by steroids has been observed so far [Isacke et al., 1989].

Although $p36_2p11_2$ is able to induce the fusion of membrane vesicles in vitro [Drust and Creutz, 1988] an integration of p36 or parts of the molecule into the lipid bilayer has not been described. p36 as well as other annexins can easily be removed from membranes or phospholipid vesicles by the chelation of Ca^{2+} without any detergent treatment. However, synexin, a recently identified member of the annexin family, is reported to act as a voltage-dependent Ca^{2+}-channel in phospholipid bilayers. Interestingly, synexin, which also comprises the four typical repeat motifs, contains an unusually long N-terminal tail [Burns et al., 1989]. Although this tail is rather hydrophobic, the four repeat motifs of synexin are proposed to form the ion channel. However, in the case of p36 or other annexins such a model is not backed by any experimental data.

p36 is an important intracellular substrate for different protein kinases. Since these phosphorylation events indicate that p36 might participate in signal transduction, (a) crucial cellular function(s) of p36 might have been overlooked so far. However, owing to the detailed biochemical and structural knowledge of the p36 molecule, we are in a position to search for those func-

tions. In this respect, it is interesting to note that a small but biochemically well-characterized portion of the p36 chain has recently been shown to be involved in a specific cellular transformation process. Mitsunobu et al. [1989] described that fusion of the N-terminal 16 residues of p36 with the c-*raf*-1 kinase domain could lead to oncogenic activation of c-*raf*-1. Interestingly, this N-terminal portion of the p36 tail harbors the complete p11 binding site but does not contain any of the major phosphorylation sites (see Fig. 1). Thus, induction of the c-*raf*-1 transforming activity in the p36-raf fusion protein of 44 KD might occur through p11-mediated formation of $p44_2p11_2$ and/or $p44p36p11_2$ complexes, which may lead to an unusual intracellular localization of the c-*raf*-1 kinase activity. Since this event could place the serine/threonine specific *raf*-kinase in close proximity to p36, it is tempting to speculate that p36 might become phosphorylated during the transformation induced by the p36-raf fusion-protein. Future experiments have to clarify whether this is indeed the case and whether an important cellular function of p36 is critically altered in *raf*-transformed cells.

REFERENCES

Burgoyne, R.D., and Geisow, M.J. (1989) The annexin family of calcium-binding proteins. Cell Calcium 10:1–10.

Burns, A.L., Magendzo, K., Shirvan, A., Srivastava, M., Rojas, E., Aluani, M.R., and Pollard, H.B. (1989): Calcium channel activity of purified human synexin and structure of the human synexin gene. Proc. Natl. Acad. Sci. U.S.A. 86:3798–3802.

Crompton, M.R., Moss, S.E., and Crumpton, M.J. (1989): Diversity in the lipocortin/calpactin family. Cell 55:1–3.

Davidson, F.F., Dennis, E.A., Powell, M., and Glenney, J.R., Jr. (1987): Inhibition of phospholipase A_2 by "lipocortins" and calpactins. J. Biol. Chem. 262:1698–1705.

Drust, D.S., and Creutz, C.E. (1988): Aggregation of chromaffin granules by calpactin at micromolar levels of calcium. Nature 331:88–91.

Erikson, E., and Erikson, R.L. (1980): Identification of a cellular protein substrate phosphorylated by the avian sarcoma virus transforming gene product. Cell 21:829–836.

Erikson, E., Tomasiewicz, H.G., and Erikson, R.L. (1984): Biochemical characterization of a 34-kilodalton normal cellular substrate of $pp60^{v\text{-}src}$ and an associated 6-kilodalton protein. Mol. Cell. Biol. 4:77–85.

Geisow, M.J., Fritsche, U., Hexham, M.J., Dash, B., and Johnson, T. (1986): A consensus amino-acid sequence repeat in Torpedo and mammalian Ca^{2+}-dependent membrane binding proteins. Nature 320:636–638.

Gerke, V., and Weber, K. (1984): Identity of p36K phosphorylated upon Rous sarcoma virus transformation with a protein purified from brush borders; Calcium dependent binding to non-erythroid spectrin and F-actin. EMBO J. 3:227–233.

Gerke, V., and Weber, K. (1985a): Calcium dependent conformational changes in the 36-kDa subunit of intestinal protein I related to the cellular 36-kDa target of Rous sarcoma virus tyrosine kinase. J. Biol. Chem. 260:1688–1695.

Gerke, V., and Weber, K. (1985b): The regulatory chain in the p36-kd substrate complex of viral tyrosine-specific protein kinases is related in sequence to the S-100 protein of glial cells. EMBO J. 4:2917–2920.

Glenney, J.R., Jr. (1986): Phospholipid-dependent Ca^{2+}-binding by the 36-kDa tyrosine kinase substrate (calpactin) and its 33-kDa core. J. Biol. Chem. 261:7247–7252.

Glenney, J.R., Jr., and Tack, B.F. (1985): Aminoterminal sequences of p36 and associated p10: Identification of the site of tyrosine phosphorylation and homology with S-100. Proc. Natl. Acad. Sci. U.S.A. 82:7884–7888.

Gould, K.L., Woodgett, J.R., Isacke, C.M., and Hunter, T. (1986): The protein-tyrosine kinase substrate, p36, is also a substrate for protein kinase C in vivo and in vitro. Mol. Cell. Biol. 6:2738–2744.

Huang, K., Wallner, B.P., Mattaliano, R.J., Tizard, R., Burne, C., Frey, A., Hession, C., McGray, P., Sinclair, L.K., Chow, E.P., Browning, J.L., Ramachandran, K.L., Tang, J., Smart, J.E., and Pepinsky, R.B. (1986): Two human 35 kd inhibitors of phospholipase A_2 are related to substrates of $pp60^{v\text{-}src}$ and the epidermal growth factor receptor/kinase. Cell 46:191–199.

Huang, K., McGray, P., Mattaliano, R.J., Burne, C., Chow, E.P., Sinclair, L.K., and Pepinsky, R.B. (1987): Purification and characterization of proteolytic fragments of lipocortin I that inhibit phospholipase A_2. J. Biol. Chem. 262:7639–7645.

Isacke, C.M., Lindberg, R.A., and Hunter, T. (1989): Synthesis of p36 and p35 is increased when U-937 cells differentiate in culture but expression is not inducible by glucocorticoids. Mol. Cell. Biol. 9:232–240.

Johnsson, N., Van Nguyen, P., Söling, H.-D., and Weber, K. (1986): Functionally distinct serine phosphorylation sites of p36, the cellular substrate of retroviral protein kinase; differential inhibition of reassociation with p11. EMBO J. 5:3455–3460.

Johnsson, N., Marriott, G., and Weber, K. (1988a): p36, the major cytoplasmic substrate of src tyrosine protein kinase, binds to its p11 subunit via a short amino-terminal amphiphatic helix. EMBO J. 7:2435–2442.

Johnsson, N., Johnsson, K., and Weber, K. (1988b): A discontinuous epitope on p36, the major substrate of src tyrosine-protein-kinase, brings the phosphorylation site into the neighbourhood of a consensus sequence for Ca^{2+}/lipid-binding proteins. FEBS Lett. 236:201–204.

Klee, C.B. (1988): Ca^{2+}-dependent phospholipid- (and membrane-) binding proteins. Biochemistry 27:6645–6653.

Kretsinger, R.H. (1987): Calcium coordination and calmodulin fold: Divergent versus convergent evolution. Cold Spring Harbor Symp. Quant. Biol. 52:499–510.

Mangeat, P.H., and Burridge, K. (1984): Immunoprecipitation of nonerythrocyte spectrin within live cells following microinjection of specific antibodies: Relation to cytoskeletal structures. J. Cell Biol. 98:1363–1377.

Mitsunobu, F., Fukui, M., Oda, T., Yamamoto, T., and Toyoshima, K. (1989): A mechanism of c-raf-1 activation: Fusion of the lipocortin II amino-terminal sequence with the c-raf-1 kinase domain. Oncogene 4:437–442.

Nigg, E.A., Sefton, B.M., Hunter, T., Walter, G., and Singer, S.J. (1982): Immunofluorescent localization of the transforming protein of Rous sarcoma virus with antibodies against a synthetic src peptide. Proc. Natl. Acad. Sci. U.S.A. 79:5322–5326.

Osborn, M., Johnsson, N., Wehland, J., and Weber, K. (1988): The submembraneous location of p11 and its interaction with the p36 substrate of pp60 src kinase *in situ*. Exp. Cell Res. 175: 81–96.

Powell, M.A., and Glenney, J.R. (1987): Regulation of calpactin I

phospholipid binding by calpactin light-chain binding and phosphorylation by $p60^{v\text{-}src}$. Biochem. J. 247:321–328.

Radke, K., and Martin, G.S. (1979): Transformation by Rous sarcoma virus: Effects of src gene expression on the synthesis and phosphorylation of cellular polypeptides. Proc. Natl. Acad. Sci. U.S.A. 76:5212–5216.

Radke, K., Gilmore, T., and Martin, G.S. (1980): Transformation by Rous sarcoma virus: A cellular substrate for transformation-specific protein phosphorylation contains phosphotyrosine. Cell 21:821–828.

Rohrschneider, L.R. (1980): Adhesion plaques of Rous sarcoma virus-transformed cells contain the src gene product. Proc. Natl. Acad. Sci. U.S.A. 77:3514–3518.

Saris, C.J.M., Tack, B.F., Kristensen, T., Glenney, J.R., Jr., and Hunter, T. (1986): The cDNA sequence for the protein-tyrosine kinase substrate p36 (calpactin I heavy chain) reveals a multidomain protein with internal repeats. Cell 46:201–212.

Schlaepfer, D.D., and Haigler, H.T. (1987): Characterization of Ca^{2+}-dependent phospholipid binding and phosphorylation of lipocortin I. J. Biol. Chem. 262:6931–6937.

Semich, R., Gerke, V., Robenek, H., and Weber, K. (1989): The p36 substrate of $pp60^{src}$ kinase is located at the cytoplasmic surface of the plasma membrane of fibroblasts: An immunoelectron microscopic analysis. Eur. J. Cell Biol., in press.

Zokas, L., and Glenney, J.R., Jr. (1987): The calpactin light chain is tightly linked to the cytoskeletal form of calpactin I: Studies using monoclonal antibodies to calpactin subunits. J. Cell Biol. 105:2111–2121.