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Regulation of calpactin I phospholipid binding by calpactin I light-chain binding and phosphorylation by $p60^{v\text{-}src}$

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Calpactins I and II are proteins that bind Ca^{2+}, phospholipids, actin and spectrin; they are also major substrates of oncogene and growth-factor-receptor tyrosine kinases. Since calpactins have been proposed to provide a link between membrane lipids and the cytoskeleton, we examined in detail the interactions between purified calpactin I and phospholipid liposomes. We focused on the Ca^{2+}-dependence, the effects of phosphorylation of calpactin I by $p60^{v\text{-}src}$ (the protein kinase coded for by the Rous-sarcoma-virus oncogene), and the effects of the binding of calpactin I light chain to calpactin I heavy chain. Binding of the light chain to the heavy chain increased the affinity of calpactin I for phosphatidylserine (PS) liposomes. The opposite effect was observed for phosphorylation by $p60^{v\text{-}src}$; phosphorylation decreased the affinity of calpactin I for PS liposomes. These two opposite effects appeared to be independent, since phosphorylation did not prevent light-chain binding to the heavy chain. Calpactin I was found, by the use of three different techniques, to bind to phospholipid liposomes at $< 10^{-8}$ M free Ca^{2+}. This result is in contrast with those of previous studies, which indicated that $> 10^{-6}$ M free Ca^{2+} was required. Our findings suggest that calpactin I may be bound to phospholipids *in vivo* at Ca^{2+} concentrations of about 1.5×10^{-7} M, typical of resting unstimulated cells, and that this interaction may be modulated by light-chain binding and phosphorylation by $p60^{v\text{-}src}$.

INTRODUCTION

Calpactins were first discovered as major substrates of oncogene and growth-factor-receptor tyrosine kinases, and it was suggested that phosphorylation of calpactins might be a key event in transformation and signal transduction by these enzymes (Radke & Martin, 1979; Erikson & Erikson, 1980; for reviews, see Cooper & Hunter, 1983; Hunter & Cooper, 1985). Calpactin I was initially referred to as 'p36', 'p34' and 'p39'; calpactin II was first called 'p35'. Both calpactins are components of membrane-associated cytoskeletons in a variety of cells, where they co-localize with non-erythroid spectrin (Cheng & Chen, 1981; Courtneidge *et al.*, 1983; Greenberg & Edelman, 1983; Lehto *et al.*, 1983). Recent biochemical studies have suggested calpactins might be involved in membrane–cytoskeleton interactions, since they bind phospholipids, actin and spectrin (Gerke & Weber, 1984; Glenney, 1986*a*,*b*; Johnsson *et al.*, 1986*a*; Glenney *et al.*, 1987).

Recent results on primary structure, antibody cross-reactivity, and functional properties have demonstrated that calpactins are related to a number of other proteins. Calpactins are identical with inhibitors of phospholipase A_2 (Huang *et al.*, 1986; Kristensen *et al.*, 1986; Saris *et al.*, 1986; Wallner *et al.*, 1986). However, subsequent studies have revealed that this inhibitory activity is due to interaction of calpactins with phospholipid substrates rather than a direct effect on phospholipase A_2 (Davidson *et al.*, 1987). The calpactin I light chain was found to share 50% sequence homology with S-100 (brain Ca^{2+}-binding proteins) (Gerke & Weber, 1985; Glenney & Tack, 1985). Calpactins are also related to proteins involved in Ca^{2+} binding and Ca^{2+}-stimulated membrane interactions, such as secretory-vesicle release (Geisow, 1986; Geisow & Walker, 1986; Geisow *et al.*, 1986; Smith & Dedman, 1986; Glenney *et al.*, 1987). Furthermore, calpactins are related to proteins induced during cell spreading in culture (Carter *et al.*, 1986; Rocha *et al.*, 1986). Calpactins also share some properties with protein kinase C; calpactins bind acidic phospholipid and Ca^{2+} (Glenney, 1986*a*), and protein kinase C exhibits activation dependent on acidic phospholipids and Ca^{2+} [reviewed by Nishizuka (1984) and Bell (1986)].

Two different calpactins have been found to co-exist in a variety of cell types: calpactin I and calpactin II (Glenney, 1986*b*; Glenney *et al.*, 1987). Calpactin I is a protein that occurs in cells as a 38 kDa monomer (the heavy chain) and as a 90 kDa complex containing two copies of the 38 kDa heavy chain and two copies of an 11 kDa light chain (Erikson *et al.*, 1984; Gerke & Weber, 1984; Glenney & Tack, 1985). Calpactin II is a 39 kDa

Abbreviations used: $p60^{v\text{-}src}$, the protein kinase coded for by the Rous-sarcoma-virus oncogene; PAGE, polyacrylamide-gel electrophoresis; PS, phosphatidylserine; PI, phosphatidylinositol; PE, phosphatidylethanolamine; PC, phosphatidylcholine.

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protein found only as a monomer (Fava & Cohen, 1984; Glenney, 1986*b*). Both calpactins can be cleaved by limited proteolysis to yield a 35 kDa 'core' and a 3 kDa 'tail' (Glenney & Tack, 1985; Glenney, 1986*a*; Johnsson *et al.*, 1986*a*; Glenney *et al.*, 1987). The 35 kDa proteinase-resistent core region contains binding sites for calcium and for acidic phospholipids (Glenney, 1986*a*; Johnsson *et al.*, 1986*a*; Glenney *et al.*, 1987). Unlike classical Ca^{2+}-binding proteins, the Ca^{2+}-dependence of this Ca^{2+} binding was shifted from millimolar to micromolar by the addition of phospholipids (Glenney, 1986*a*). The phospholipid binding of this site has appeared to require micromolar Ca^{2+} (Fava & Cohen, 1984; Glenney, 1986*a*; Johnsson *et al.*, 1986*a*; Glenney *et al.*, 1987). The 3 kDa tail represents the *N*-terminal region of intact calpactin I. The tail contains the tyrosine phosphorylation sites on both calpactins (Glenney & Tack, 1985; De *et al.*, 1986) and the binding site on the calpactin I heavy chain for the 11 kDa calpactin I light chain (Glenney *et al.*, 1986; Johnsson *et al.*, 1986*a*). Owing to the presence of the phosphorylation site and light-chain binding site, the tail region has been proposed as the regulatory portion of the proteins (Glenney *et al.*, 1986; Johnsson *et al.*, 1986*a*).

Because of the potential importance of the phospholipid binding, we have examined further the phospholipid-binding properties of calpactin I, including the effects of phosphorylation and light-chain binding, and the Ca^{2+}-dependence. We have found that phosphorylation decreased phospholipid binding, and that light-chain binding increased phospholipid binding. We have also found that calpactin I was bound to acidic phospholipids at very low free-Ca^{2+} concentrations ($< 10^{-8}$ M).

MATERIALS AND METHODS

Calpactins were purified as described by Glenney *et al.* (1987). Phospholipid binding was monitored two ways: by co-sedimentation in an ultracentrifuge as described by Glenney (1985) and by sucrose flotation gradients as described by Davidson *et al.* (1987). Phospholipid aggregation was monitored as described by Glenney *et al.* (1987). Liposomes were prepared by sonication (with a probe-type sonicator) of phospholipid suspensions until they were clear. All solutions with measured concentrations of Ca^{2+} contained EGTA as a Ca^{2+} buffer. Ca^{2+} concentrations were measured using an Orion Ca^{2+} sensitive ion electrode, according to instructions provide by the manufacturer. Since Ca^{2+} concentrations below about 0.5 μM could not be measured accurately, these levels were estimated by calculation using dissociation constants of EGTA for Ca^{2+} and Mg^{2+} at the relevant pH (Caldwell, 1970). Sucrose-gradient analysis of light-chain binding to calpactin I monomer was performed as described by Glenney *et al.* (1986).

Calpactin I was phosphorylated by $p60^{v\text{-}src}$ as described by Glenney (1985). The phosphorylation showed a low stoichiometry, owing to the low activity of our $p60^{v\text{-}src}$ preparation; tracer amounts of ^{32}P were used (the exact stoichiometry was not determined). Comparison of the properties of phosphorylated against unphosphorylated calpactin I was performed by a comparison of the behaviour of the ^{32}P-labelled calpactin I against the behaviour of the calpactin I detected by protein staining. The calpactin I detected by protein staining included phosphorylated calpactin I, but the proportion of calpactin which was phosphorylated was so low that it was an insignificant fraction of the total. For these comparisons, samples from phospholipid-binding assays of sucrose-gradient fractions were analysed by SDS/PAGE. The gels were first stained with Coomassie Blue and calpactin I in each lane was quantified by densitometry; then the calpactin I bands in each lane were exised and the [^{32}P]calpactin I was quantified by liquid-scintillation counting.

RESULTS

Ca^{2+} independent binding of calpactin I to PS liposomes

Earlier studies of calpactin interaction with phospholipids demonstrated Ca^{2+}-dependent binding. More detailed studies now show that up to 80% of the calpactin I complex was bound to PS liposomes at free-Ca^{2+} concentrations below 10^{-8} M in EGTA buffers (low Ca^{2+}). We found variability in our early studies, but this variability could be greatly decreased by consistently using liposomes prepared the same day as the binding experiments. An example of binding data obtained with one batch of freshly prepared liposomes is shown in Fig. 1. About 70% of calpactin I complex bound to PS liposomes at free-Ca^{2+} concentrations below 10^{-8} M, whereas calpactin I monomer apparently required Ca^{2+} at $\geqslant 10^{-5}$ M for $> 50\%$ binding. However, even with freshly prepared liposomes, some variability was observed; calpactin I complex binding ranged from 60 to 80% at low [Ca^{2+}], and the [Ca^{2+}] required for 50% binding of calpactin I monomer varied from about 10 μM to 20 μM.

Since variability in the amount of exposed PS might account for differences in binding, the phospholipid-concentration-dependence of binding was measured with

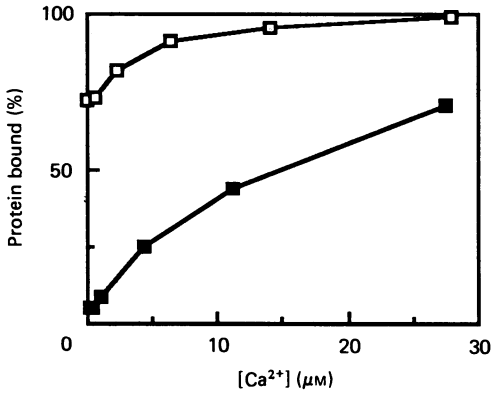

Fig. 1. Ca^{2+}-dependence of binding of calpactin I to PS liposomes

Proteins (200 μg/ml) were mixed with PS liposomes (250 μg/ml), left at room temperature for 30 min, and centrifuged for 20 min at 175000 *g* at room temperature. Buffers contained 10 mM-EGTA and various amounts of free Ca^{2+}. Supernatants were removed, and supernatants and pellets were made up to equal final volume in sample buffer. Equal volumes of supernatant and pellet were run in adjacent lanes on SDS-containing gels, stained with Coomassie Blue, and calpactin bands were quantified with a densitometer. □, Calpactin I complex; ■, calpactin I monomer.

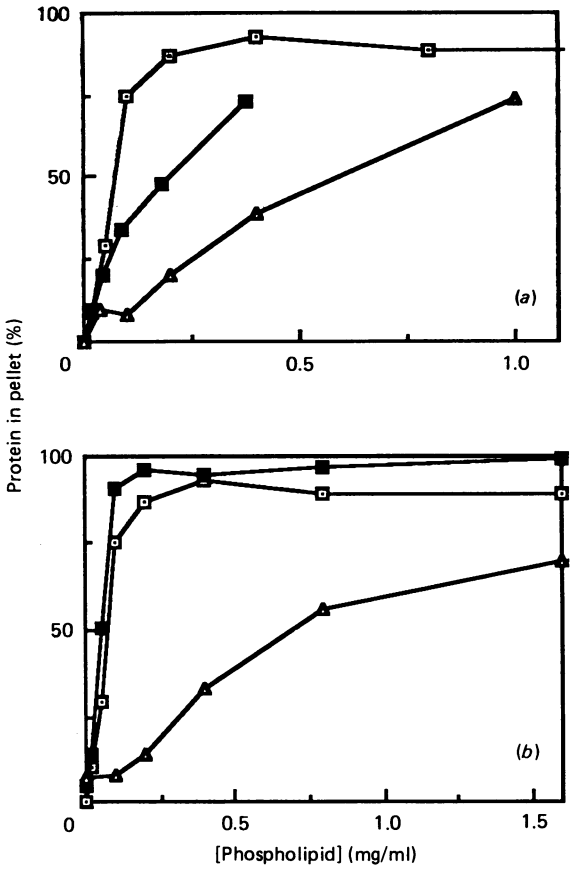

Fig. 2. PS-concentration-dependence of binding of calpactin I to PS liposomes at $< 10^{-8}$ M-Ca^{2+}

Assays were performed as described in the legend to Fig. 1. (*a*) Binding of calpactin I complex to three different liposome preparations. □ and ■, different liposome preparations which were used the same day as they were prepared; △, liposomes prepared and stored at 4 °C for 1 month before use. All liposomes were prepared by identical methods. (*b*) Binding to one batch of freshly prepared liposomes of calpactin I complex and calpactin I monomer. ⊡, calpactin I complex; △, calpactin I monomer at < 0.5 μM-Ca^{2+}; ■, calpactin I complex at 4 μM-free Ca^{2+}.

several different preparations of liposomes (Fig. 2*a*). The concentration of PS required for 50% binding of calpactin I complex varied over a 2-fold concentration range for two different batches of freshly prepared liposomes. When liposomes prepared 1 month before the assay were used, the concentration of PS required for 50% binding of calpactin I complex was several times higher than for fresh liposomes. Fig. 2(*b*) shows the results of binding experiments for one batch of liposomes using calpactin I complex at low [Ca^{2+}] and at 4 μM-Ca^{2+}, and calpactin I monomer at low [Ca^{2+}]. The PS level required for 50% binding of the complex was 10-fold lower than for 50% binding of the monomer at low [Ca^{2+}]. Nevertheless, at high phospholipid concentrations, the monomer was bound to the liposomes at low [Ca^{2+}]. The complex bound to PS liposomes about equally at the two different Ca^{2+} concentrations.

In contrast with previous studies, which showed that Ca^{2+} concentrations above 10^{-6} M were required for binding of calpactin I to PS liposomes, our results shown above demonstrated binding of calpactin I to PS liposomes at $< 10^{-8}$ M-Ca^{2+} (low [Ca^{2+}]). To study further this discrepancy, we used additional techniques to measure binding at low [Ca^{2+}]. Fig. 3 shows the results of using sucrose flotation gradients. In these gradients, liposomes with or without proteins were loaded in 60% sucrose with a 50% sucrose overlay, all in EGTA-containing buffers. After centrifugation, the liposomes, together with bound proteins, were found at the top of the gradient. Unbound proteins remained at the bottom of the gradient in the 60% sucrose, where they were loaded. Calpactin I complex floated with the liposomes at low [Ca^{2+}] at the higher phospholipid concentration used (Fig. 3*b*), but not at the lower phospholipid concentration (Fig. 3*a*). Calpactin I monomer did not float at either lipid concentration (Figs. 3*c* and 3*d*).

Since calpactins aggregate phospholipids, measurement of the Ca^{2+}-dependence of this phenomenon may be another way to demonstrate binding of calpactins to phospholipids at low [Ca^{2+}]. Fig. 4 shows the results of light-scattering assays to monitor aggregation of phospholipids by calpactin I at various concentrations of Ca^{2+} (aggregation caused increased absorbance). Calpactin I complex bound to, and aggregated, PS liposomes at $< 10^{-8}$ M-Ca^{2+}, whereas calpactin I monomer did not aggregate PS liposomes at low [Ca^{2+}]. These results demonstrate binding of calpactin I complex to PS liposomes at low [Ca^{2+}].

Effect of light chain on calpactin phospholipid binding

The results shown above demonstrate a difference between the binding of calpactin I monomer and calpactin I complex to PS liposomes. Fig. 5 shows the results of an experiment designed to test the effects of added light chain on calpactin I monomer phospholipid binding. Various amounts of light chain were added to calpactin I monomer and the effects of the added light chain on phospholipid binding of calpactin I monomer were monitored. Previous studies (Glenney *et al.*, 1986) and Fig. 8 (below) demonstrated that light chain was bound to calpactin I monomer under these conditions, and a complex indistinguishable from native calpactin I complex was formed. Addition of 1 mol of light chain/mol of calpactin I monomer resulted in binding to PS liposomes equivalent to the binding of calpactin I isolated as the native complex. Further addition of light chain had no effect. The light chain alone did not bind to PS liposomes. The phospholipid binding of calpactin I complex and calpactin I core (which lacked the binding site for light chain) were both unaffected by the added light chain. Thus phospholipid binding of calpactin I monomer at $< 10^{-8}$ M-Ca^{2+} was greatly increased by the binding of light chain to the monomer, with 1 mol of light chain/mol of monomer required for maximum effect.

Effects of tyrosine phosphorylation on calpactin phospholipid binding

Since phosphorylation of calpactin I on tyrosine has been suggested to be an important regulatory event, we examined its effects on phospholipid binding by calpactin I. Table 1 shows the effects of phosphorylation of calpactin I complex by $p60^{v\text{-}src}$ on binding of calpactin I complex to liposomes of various phospholipids. Phosphorylation did not change the phospholipid specificity of

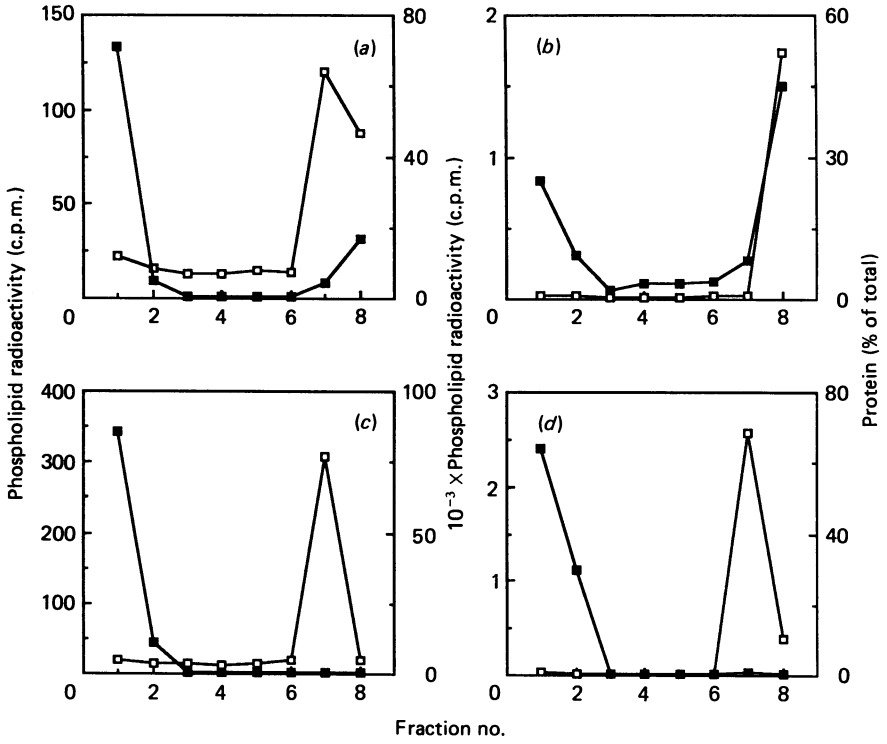

Fig. 3. Binding of calpactin I to PS liposomes at $< 10^{-8}$ M-Ca^{2+} monitored by using sucrose gradients to separate calpactin I bound to liposomes from unbound calpactin I

Proteins were mixed with [^{14}C]PS liposomes in EGTA buffer containing 60% (w/v) sucrose and left for 30 min at room temperature. Samples (100 μl) were placed at the bottom of centrifuge tubes, carefully overlayed with 50% sucrose in the same buffer and with 50 μl of buffer without sucrose. Tubes were centrifuged for 2 h at 120000 *g*. Fractions were analysed for [^{14}C]PS by liquid-scintillation counting and for protein by SDS/PAGE, Coomassie Blue staining, and quantification with a densitometer. Fraction 1 was the bottom of each gradient. (*a*) Calpactin I complex (200 μg/ml) and liposomes at 200 μg/ml; (*b*) calpactin I complex (200 μg/ml) and liposomes at 1 mg/ml; (*c*) calpactin I monomer (200 μg/ml) and liposomes at 200 μg/ml; (*d*) calpactin I monomer (200 μg/ml) and liposomes at 1 mg/ml. □, [^{14}C]PS; ■, amounts of calpactin I in each fraction (%).

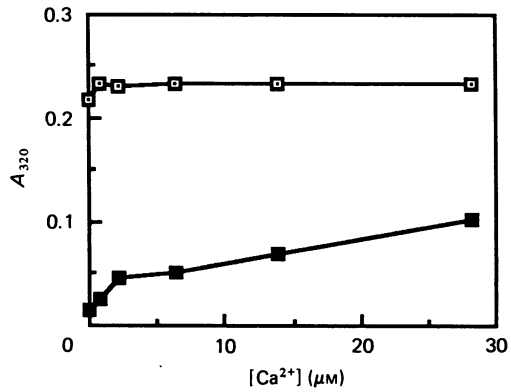

Fig. 4. Ca^{2+}-dependence of calpactin-induced aggregation of liposomes

Aggregation caused increased absorbance. Proteins were mixed with liposomes (300 μg/ml) in EGTA buffers with various concentrations of free Ca^{2+}, left for 30 min at room temperature, and the absorbance was measured at 320 nm after gentle mixing. □, Calpactin I complex at 40 μg/ml; ■, calpactin I monomer at 20 μg/ml. Protein concentrations used were the minimum amounts of protein required to produce complete aggregation of the liposomes at 1 mM free Ca^{2+} as determined by a separate experiment.

calpactin I complex binding to liposomes. Phosphorylation slightly decreased the binding of calpactin I complex to PS and phosphatidylinositol (PI) at both high and low [Ca^{2+}] and did not induce binding of calpactin I complex to phosphatidylethanolamine (PE) and phosphatidylcholine (PC).

Phosphorylation appeared to decrease the phospholipid binding of calpactin I complex, so we measured the effects of phosphorylation on the amount of calpactin I complex bound at various phospholipid concentrations. Fig. 6 shows the effect of phosphorylation on the phospholipid-concentration-dependence of calpactin I complex binding to PS liposomes at $< 10^{-8}$ M-Ca^{2+}. The concentration of phospholipid required for 50% binding was increased by nearly a factor of 2 for the phosphorylated form of calpactin I complex compared with the unphosphorylated form at low [Ca^{2+}]. In addition, less of the phosphorylated form was bound at all phospholipid concentrations tested.

Fig. 7 shows the effects of phosphorylation on the Ca^{2+}-dependence of calpactin I phospholipid binding. Phosphorylation decreased binding of calpactin I to PS liposomes at all Ca^{2+} concentrations tested. This effect was observed for calpactin I complex, calpactin I monomer and calpactin I complex reconstituted by

addition of light chain to previously phosphorylated calpactin I monomer.

Since phosphorylation appeared to decrease the affinity of calpactin for phospholipid, and addition of light chain increased the phospholipid binding of calpactin I, we tested whether the change due to phosphorylation was caused by decreased binding of light chain to calpactin I monomer. Fig. 8 shows the results of using sucrose gradients, which measured the size of phosphorylated and unphosphorylated calpactin I monomer when light chain was added. When light chain was added, both the unphosphorylated and phosphorylated calpactin I monomer migrated on the gradients to a position corresponding to native calpactin I complex. These results indicated that both phosphorylated and unphosphorylated monomer bound light chain.

DISCUSSION

Changes in intracellular Ca^{2+} concentrations are thought to regulate a number of cellular activities. These processes are typically mediated by Ca^{2+}-binding proteins, such as calmodulin and protein kinase C. Calmodulin binds to, and regulates, the activity of a variety of enzymes in a Ca^{2+}-dependent manner (Cheung, 1980). In contrast, protein kinase C shows a different mode of action (Nishizuka, 1984; Bell, 1986). Ca^{2+} signals mediated by C kinase result in translocation of C kinase to the membrane, where it binds acidic phospholipids and diacylglycerol and phosphorylates target proteins.

Calpactins are members of another class of Ca^{2+}-binding proteins whose role in signal transduction by Ca^{2+} is unclear (Geisow & Walker, 1986; Geisow *et al.*, 1986). Previous studies have shown that Ca^{2+} concentrations above 10^{-6} M were required for phospholipid binding of calpactin I (Glenney, 1986*a*; Johnsson *et al.*,

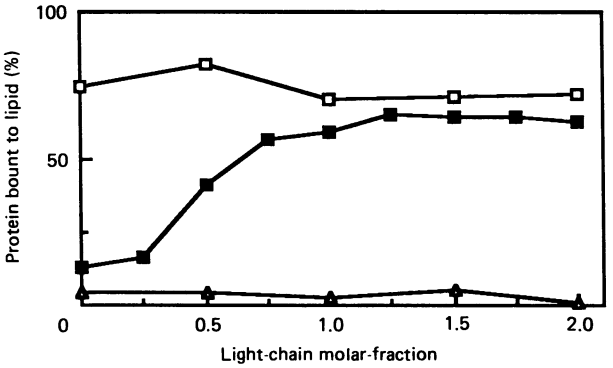

Fig. 5. Effect of light chain on binding of calpactin I monomer to PS liposomes at $< 10^{-8}$ M-Ca^{2+}

Binding assays were performed as described in the legend to Fig. 1. Various amounts of calpactin I light chain were mixed with 200 μg of calpactin I complex (□), calpactin I monomer (■) or calpactin I core (△)/ml, and left 30 min at room temperature. Proteins were then mixed with PS liposomes (200 μg/ml) and left for an additional 30 min before centrifugation.

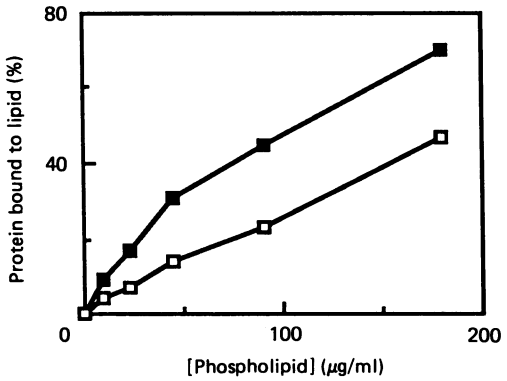

Fig. 6. Phospholipid-concentration-dependence of binding of phosphorylated and unphosphorylated calpactin I complex to PS liposomes at $< 10^{-8}$ M-Ca^{2+}

Assays were performed as described in the legend to Fig. 1. Proteins were phosphorylated with tracer amounts of [^{32}P]ATP by p60$^{v\text{-}src}$ as outlined in the Materials and methods section. Calpactin I complex was mixed with various amounts of PS liposomes and the amount of phosphorylated and unphosphorylated calpactin I bound to the liposomes was determined as described in the legend to Table 1. □, Phosphorylated calpactin I complex; ■, unphosphorylated calpactin I complex.

Table 1. Binding of phosphorylated and unphosphorylated calpactin I complex to liposomes of various phospholipids at $< 10^{-8}$ M free calcium (low) and 40 μM free calcium (high)

Proteins were phosphorylated with tracer amounts of [^{32}P]ATP by p60$^{v\text{-}src}$ as outlined in the Materials and methods section. The partially phosphorylated calpactin was mixed with liposomes in EGTA-containing buffers, left for 30 min at room temperature, centrifuged for 20 min at 175000 *g*, and the supernatant was removed. Sample buffer was added to the supernatant and the pellet was dissolved in sample buffer to equal to the supernatant volume. Equal volumes of supernatant and pellet were run in adjacent lanes of SDS-containing gels. The gels were stained with Coomassie Blue, and calpactin bands were quantified with a densitometer. The calpactin I band was excised from each lane of the gel and [^{32}P]calpactin in each lane was determined by liquid-scintillation counting.

		Percentage bound							
	Phospholipid ...	PS		PI		PE		PC	
Complex	[Ca^{2+}] ...	Low	High	Low	High	Low	High	Low	High
Calpactin I complex		70	98	54	85	1	3	1	1
Phosphorylated calpactin I complex		42	84	38	62	0	1	0	0

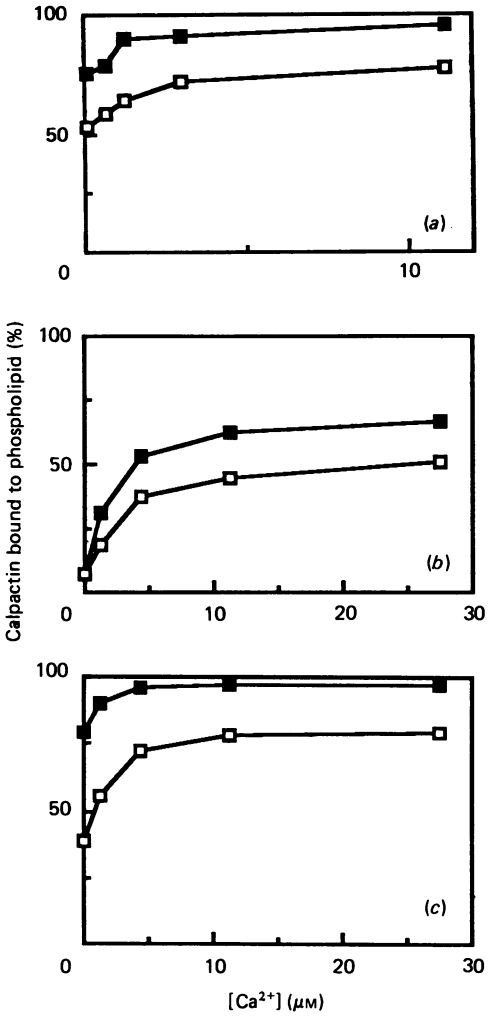

Fig. 7. Calcium dependence of binding of phosphorylated and unphosphorylated calpactin I to PS liposomes

(*a*) Calpactin I complex; (*b*) calpactin I monomer; (*c*) calpactin I monomer was phosphorylated, then 1 mol of light chain per mol of calpactin I monomer was added, the mixture left for 30 min at room temperature, then the binding assay was performed as described in the legend to Table 1. □, Phosphorylated calpactin I; ■, unphosphorylated calpactin I.

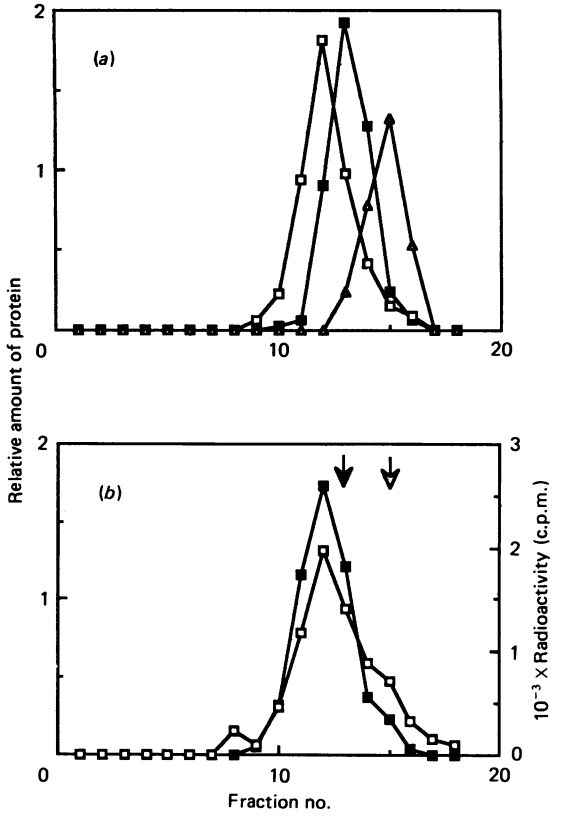

Fig. 8. Analysis of light-chain binding to phosphorylated and unphosphorylated calpactin I monomer by sucrose gradients

Proteins were layered over 5–20% (w/v) sucrose gradients and centrifuged for 18 h at 40000 rev./min in a Beckman SW-60 rotor at 4 °C. In all, 20 fractions were collected and analysed by SDS/PAGE and Coomassie Blue staining. Proteins were quantified by densitometry, and phosphorylated proteins were quantified by excision of calpactin bands from the gels and liquid-scintillation counting. (*a*) Calpactin I complex, calpactin I core, and bovine serum albumin were layered on the gradient together. □, Calpactin I complex; ■, bovine serum albumin; △, calpactin I core. (*b*) Phosphorylated and unphosphorylated calpactin I monomer were mixed with 1 mol of light chain/mol of calpactin I monomer, left for 30 min at room temperature and then loaded on the gradient with bovine serum albumin as an internal standard. ↓, bovine serum albumin; ↓, predicted position of calpactin I monomer. □, phosphorylated calpactin I; ■, unphosphorylated calpactin I.

1986*a*; Glenney *et al.*, 1987). This result suggested that phospholipid binding might occur (if ever) only in cells undergoing sharply elevated Ca^{2+} transients, such as stimulated muscle cells, stimulated secretory cells or cells stimulated by growth factors (Williams *et al.*, 1985; Moolenaar *et al.*, 1986). However, our results demonstrate that calpactin I was bound to phosphatidylserine liposomes at $< 10^{-8}$ M-Ca^{2+} (low [Ca^{2+}]). This phospholipid binding was probably Ca^{2+}-independent, since direct Ca^{2+}-binding experiments showed virtually no Ca^{2+} binding below 10^{-6} M-Ca^{2+} under the same conditions (Glenney, 1986*a*,*b*). These experiments *in vitro* cannot answer the question of whether calpactin I is bound to phospholipids *in vivo*, since the physical state of the phospholipids *in vivo* is likely to be different. However, the Ca^{2+}-independent binding shown here suggests calpactin I may be bound to phospholipids *in vivo* at Ca^{2+} concentrations of about 1.5×10^{-7} M, characteristic of resting unstimulated cells (Williams *et al.*, 1985; Moolenaar *et al.*, 1986).

Since our findings of calpactin phospholipid binding at low [Ca^{2+}] were different from previous results, we used three different techniques to monitor this interaction: sedimentation, flotation and light-scattering. All three showed binding at low [Ca^{2+}]. The apparent contradiction between our findings and previous results may be explained by the phospholipid levels used for the assays. Indeed, the sucrose-gradient binding assays shown in Fig. 3 demonstrated this point. Fig. 3(*a*) duplicated a previously published experiment (Davidson *et al.*, 1987), and neither of these experiments revealed binding of

calpactin I complex to liposomes at low [Ca^{2+}]. The sucrose gradient shown in Fig. 3(*b*) was also identical, except that a higher phospholipid concentration was used, and most of the calpactin I complex was bound to the liposomes under these conditions. Thus previous studies demonstrating a Ca^{2+}-dependence in the micromolar range for phospholipid binding of calpactins and related proteins might have generated a different result if higher phospholipid concentrations had been used; they might have shown binding of calpactin I to phospholipids at low [Ca^{2+}] (Fava & Cohen, 1984; Glenney, 1986*a*; Johnsson *et al.*, 1986*a*; Glenney *et al.*, 1987; related proteins reviewed in Geisow & Walker, 1986).

The present results also demonstrate both positive and negative regulation of phospholipid binding by calpactin I. This regulation was probably caused by changes in the affinity of calpactin I for phospholipids. We have not attempted to determine the affinity of these proteins for phospholipids, because of the complexity of the mixture; binding of calpactins to phospholipids caused aggregation such that the phospholipids and calpactin settled out of solution without centrifugation. However, we measured the amounts of phospholipids required for half-maximal binding of calpactin I to the phospholipids. Changes in the phospholipid requirements for half-maximal binding are probably reflective of changes in the affinity of calpactin I for phospholipids.

The *N*-terminal 'tail' region of calpactin I is responsible for regulation of the phospholipid binding of calpactin I. This tail region contains the binding site for the calpactin I light chain (Glenney *et al.*, 1986; Johnsson *et al.*, 1986*a*,*b*), and the site of tyrosine phosphorylation by $p60^{v\text{-}src}$ (Glenney & Tack, 1985). Light-chain binding had a stimulatory effect on phospholipid binding, and phosphorylation had an inhibitory effect on phospholipid binding. The phospholipid-binding sites were on the 35 kDa *C*-terminal core region of calpactin I (Glenney, 1986*a*; Johnsson *et al.*, 1986*a*), so it appeared that the 3 kDa tail could regulate properties attributed to the core region of the protein. The regulation by phosphorylation and light chain may be independent, since phosphorylation did not prevent light-chain binding.

Several lines of evidence suggest light-chain binding had a stimulatory effect on phospholipid binding. First, calpactin I complex showed a higher level of binding to phospholipids than calpactin I monomer under all conditions tested (Figs. 1–4). The phospholipid requirement for 50% binding was 10-fold lower for the complex compared with the monomer (Fig. 2). This effect is interpreted as a 10-fold higher affinity for phospholipids of calpactin I complex compared with calpactin I monomer. Second, addition of calpactin I light chain to calpactin I monomer in various amounts revealed increased binding to phospholipids; the increase was in proportion to the amount of light chain added, with a maximum effect at 1 mol of light chain/mol of monomer (Fig. 5). Controls showed that light chain had no effect on phospholipid binding of calpactin I core which had lost its light-chain binding site (Glenney *et al.*, 1986).

The inhibitory effect of phosphorylation on phospholipid binding was demonstrated by several experiments. First, phosphorylation increased the amount of phospholipid required for 50% binding of calpactin I complex. This effect probably represented a decrease in affinity for phospholipids upon phosphorylation of calpactin I complex (Fig. 6). Second, phosphorylation decreased the amount of calpactin I bound to phospholipid under all conditions tested. This effect was observed with calpactin I monomer, calpactin I complex and calpactin I complex formed by addition of light chain to phosphorylated calpactin I monomer (Figs. 6 and 7).

Previous studies have suggested a regulatory role for the *N*-terminal tail of calpactin I might be found, since the light-chain binding site and tyrosine phosphorylation site of calpactin I are both found on the tail (Glenney, 1985; Johnsson *et al.*, 1986*a*,*b*; Glenney *et al.*, 1986). One effect found was that phosphorylation of some, but not all, serine sites on the tail decreased light-chain binding to the tail (Johnsson *et al.*, 1986*b*). However, phosphorylation by protein kinase C, which is thought to phosphorylate calpactin I *in vivo* (Gould *et al.*, 1986), did not block light-chain binding; only phosphorylation by cyclic AMP-dependent kinase and calmodulin-dependent kinase had an effect on light-chain binding. Our results show that tyrosine phosphorylation of calpactin I by $p60^{v\text{-}src}$ did not prevent light-chain binding to the tail. The position of phosphorylation may have determined whether phosphorylation blocked light-chain binding. Protein kinase C phosphorylated serine-25, $p60^{v\text{-}SRC}$ phosphorylated tyrosine-23, and these phosphorylations did not affect light-chain binding to the tail (Fig. 8; Johnsson *et al.*, 1986*b*). In contrast, phosphorylation closer to the *N*-terminus drastically decreased light-chain binding. Another suggested regulatory effect of the tail was that the presence of light chain on the tail decreased the ability of several serine/threonine kinases to phosphorylate calpactin I on the tail (Johnsson *et al.*, 1986*b*; Khanna *et al.*, 1986). However, these results are difficult to interpret, since only stoichiometry of phosphorylation was determined and no data were presented concerning the affinity or maximal rate of the kinases for calpactin I with and without light chain.

We believe the properties of calpactins shown *in vitro* may provide some clues to the functions of calpactins *in vivo*. Phosphorylation of calpactin I has been shown *in vivo*, and both calpactin I complex and calpactin I monomer co-exist *in vivo*. Thus the affinity of calpactin I for its binding site *in vivo* may be increased by the presence of calpactin I light chain or decreased by tyrosine phosphorylation. Although the target site of calpactin I *in vivo* is unclear, calpactin I may be bound to target sites in cells under typical unstimulated calcium conditions of about 1.5×10^{-7} M. If increases in Ca^{2+} concentration are not required for binding of calpactin I to phospholipids, then there may be another role for Ca^{2+} binding. One possibility is that increases in Ca^{2+} concentrations may cause a conformational change in calpactins which results in a structural change in the calpactin target sites. This type of Ca^{2+} signal transduction would be analagous to calmodulin activity, but may have a different target. These functions may be modulated by the presence of calpactin I light chain and by phosphorylation on tyrosine. However, the question of the target site of calpactins remains problematic. Calpactins have been shown to bind to spectrin, actin and phospholipids, and it is not known which, if any, of these are important *in vivo*. Until the binding sites of calpactin *in vivo* are found, the role of light-chain binding and phosphorylation will remain speculative.

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