Calbindin$_{28kDa}$ and calbindin$_{30kDa}$ (calretinin) are substantially localised in the particulate fraction of rat brain

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Abstract **Calbindin$_{28kDa}$ is implicated in cytosolic calcium transport and calcioprotection functions, principally as a mobile calcium buffer. Using immunoblotting, we have found that 36% of total calbindin$_{28kDa}$ is in the particulate fraction of rat brain. Particulate calbindin$_{28kDa}$ was located both within and outside organelles and required detergent for solubilisation. Equivalent observations were made for calbindin$_{30kDa}$, 27% of which was insoluble. These findings indicate that a substantial proportion of calbindin does not function as a mobile calcium buffer, and perhaps instead has a calcium signalling role through target ligands in the insoluble cellular fraction.**

Key words: Calbindin; Calcium-binding protein; Calcium transport; Cytotoxicity

1. Introduction

Calbindin$_{28kDa}$ is a high affinity calcium-binding protein belonging to the calmodulin superfamily (reviewed in [1,2]), for which three principal roles have been proposed. First, following its discovery through association with vitamin D-dependent calcium uptake in the avian gut, calbindin$_{28kDa}$ has received considerable attention as a mediator of transcellular calcium transport in several tissues where it is abundantly expressed (e.g. kidney, dental enamel epithelium, avian intestine and egg shell gland) [3–6]. Second, the high abundance of calbindin$_{28kDa}$ in certain neurones conflicts with a unique function in calcium transport. Investigations in these cells have instead led to purported calcioprotection (calcium buffering) roles [7–9]. A third possible function for calbindin$_{28kDa}$, as a calmodulin-like transducer of the calcium signal to target proteins, is based on the structural similarity between calbindin$_{28kDa}$ and calmodulin, and recognition that the strongly conserved features of calbindin$_{28kDa}$ extend beyond the calcium-binding loops [10,11]. While potential targets for calbindin$_{28kDa}$ are recognised [12–14], the physiological relevance of such interactions remains unclear. Calbindin$_{28kDa}$ is thought to be medically important through its associations with normal and disturbed calcium homeostasis, but the current lack of a precisely defined function obscures understanding at the molecular level. For example, calbindin$_{28kDa}$ expression has been strongly associated with some neurodegenerative disorders and apoptosis, but several inconsistencies remain [15–20].

Calbindin$_{28kDa}$ is widely regarded as a soluble protein, resident primarily in the cytosol [1,2]. Following subcellular fractionation, only 4–9% of total calbindin$_{28kDa}$ was recovered in the insoluble fractions from intestine and brain, mainly associated with the nuclear pellet [21–25]. The solubility of calbindin$_{28kDa}$ and its mobility through the cytosol are central to the putative roles as a calcium ferry or diffusible buffer [8,26]. Conversely, immunocytochemistry on several tissues has indicated that some calbindin$_{28kDa}$ is associated with subcellular structures including the nucleus, vesicles and cytoskeleton, but the proportion reported to be so localised varies considerably [4,23,25,27].

A recently discovered cognate of calbindin$_{28kDa}$, termed calbindin$_{30kDa}$ or calretinin, is expressed mainly in certain neurones [15,16,28]. The subset of calbindin$_{30kDa}$-containing neurones overlaps partially with that containing calbindin$_{28kDa}$, suggesting that these proteins have similar but distinct functions. Concordantly, the 28 kDa and 30 kDa calbindins were found to have nearly equal calcium binding properties [29]. No biochemical analysis of calbindin$_{30kDa}$ subcellular localisation has been reported to our knowledge, but an association with microtubules was detected with immunocytochemistry [30].

Using quantitative immunoblotting, we found that 33% of total (SDS-soluble) calbindin$_{28kDa}$ was located in the particulate fraction of rat enamel cells, principally associated with the Triton-insoluble cytoskeleton [31]. To evaluate whether this substantial insolubility of calbindin$_{28kDa}$ extended to another enriched tissue, and to calbindin$_{30kDa}$, the present study applied the same approach to rat brain. The results revealed a markedly higher proportion of insoluble calbindin than have previous subcellular fractionation studies, and also provided novel support for the existence of target ligands in the insoluble cellular fraction.

2. Experimental

2.1. Extracts and subcellular fractionation

Brains from 7–8-week-old Wistar-derived rats, anaesthetised with ether, were homogenised immediately or, where indicated, frozen on dry ice and stored at −80°C. Homogenates were prepared with a motorised tissue disperser (Tissue Tearor, from BioSpec) in 3 vols. ice-cold homogenisation buffer (10 mM Tris-HCl, pH 7.2, 120 mM NaCl, 5 mM dithiothreitol) containing protease inhibitors (0.1 mM phenymethanesulfonyl fluoride, 1 mM benzamidine). Samples of homogenate were ultracentrifuged for 5 min at 2×10^5 Pa ($\approx 150{,}000 \times g$) in a Beckman Airfuge and the resultant supernatant is referred to as soluble fraction or cytosol. The pellet, following one washing step of homogenising and resedimenting as above, is termed the total particulate fraction. Where indicated, fractions enriched in nuclei, mitochondria and microsomes were isolated from the homogenate (200 μl) by sequential centrifugation steps (each 5 min at 4°C) at $1{,}000 \times g$, $15{,}000 \times g$ and $150{,}000 \times g$, respectively. Before SDS-PAGE, all particulate fractions were routinely extracted with SDS denaturant (2% SDS in 10 mM Tris-HCl, pH 7.2, 2 mM dithiothreitol) containing protease inhibitors, using bath sonication and heating (5 min, 100°C), then clarified in a microcentrifuge ($17{,}000 \times g$, 2 min) at room temperature.

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2.2. Gel electrophoresis procedures

SDS-PAGE (12.5% T, 2.7% C) with the Laemmli discontinuous buffer system, denaturing two-dimensional gel electrophoresis and ^{45}Ca-overlays were as described [31,32]. The heterogeneous mobility of calcium-loaded calbindins was largely overcome by including 5 mM EGTA in the sample buffer for SDS-PAGE, but in some experiments (e.g. Fig. 1A and 4A) calcium-induced shifts were evident.

Quantitative immunoblotting was done as documented fully elsewhere [31]. Briefly, immunoblots were developed with avidin-biotin-peroxidase (diaminobenzidine substrate) and quantitated by imaging densitometry (BioRad Model GS670) under linear conditions, using HPLC-purified calbindin (quantified by amino acid analysis) as internal standard. Values obtained from this immunoassay were the same as those from calibrated ^{45}Ca-overlays run in parallel ([31] and data not shown). Statistical comparison of the mean values for calbindin immunoreactivity was done with Student's *t*-test (2-tailed, homoscedastic).

2.3. Antibodies

Antiserum to rat brain calbindin$_{30kDa}$ was raised in a New Zealand White rabbit using the emulsified and immobilised immunogen presentations and administration routes described before [32]. Using native dot blots and denaturing immunoblots of protein standards and cerebellum cytosol, the antiserum at 0.01 dilution was found to be monospecific for low nanogram amounts of calbindin$_{30kDa}$ with reactivity independent of calcium. Calbindin$_{28kDa}$ was detected with approximately 10-fold lower sensitivity under these conditions (Fig. 4 and data not shown). The calbindin$_{30kDa}$ immunogen was purified from juvenile rat cerebellum using heat treatment (65°C), size exclusion chromatography and anion exchange chromatography in the presence and absence of calcium [31]. Monospecific antiserum to rat brain calbindin$_{28kDa}$ was similarly prepared and characterised as described earlier [31].

3. Results

3.1. Subcellular fractionation of rat brain calbindin$_{28kDa}$

Quantitative immunoblot analysis of the soluble fraction (150,000 × *g* supernatant) from freshly dissected cerebellum revealed calbindin$_{28kDa}$ in high specific abundance (9 ± 2 μg·mg protein^{-1}; ± S.E.M., n = 3), consistent with data obtained using other immunoassays and ^{45}Ca-overlay analysis [31,33]. However, contrary to calbindin$_{28kDa}$ being a soluble protein, substantial immunoreactivity was also detected in the SDS-solubilised particulate fraction (Fig. 1A). A further SDS extraction of the residual pellet yielded no additional calbindin$_{28kDa}$. Densitometry indicated that, at some higher sample loadings where the soluble fraction gave a linear response, immunoreactivity of the SDS extract was non-linear leading to underestimation of particulate calbindin$_{28kDa}$. The likelihood that the nonlinearity arose from sample overloading was supported by analysis of parallel gels stained with Coomassie blue. When the pellet was instead extracted with Triton X-100, increased amounts of calbindin$_{28kDa}$ were detected and the immunoreactivity was linear. Subsequent treatment of the Triton-insoluble fraction with SDS released little additional calbindin$_{28kDa}$ (Fig. 1A). Together, particulate calbindin$_{28kDa}$ solubilised sequentially with Triton X-100 and SDS amounted to 36 ± 2% (n = 5) of the total calbindin$_{28kDa}$ detected.

To establish whether this behaviour was a feature of the cerebellum, where calbindin$_{28kDa}$ expression levels are exceptionally high, the analyses were repeated on cerebrum. Again, it was found that particulate calbindin$_{28kDa}$ accounted for over one-third (35 ± 3%, n = 3) of the total immunoreactivity (Fig. 1A). Equivalent results were also obtained with cerebellum and cerebrum samples that had been stored at −80°C.

Subfractionation of the pellet into crude nuclear, mitochondrial and microvesicular components showed that calbindin$_{28kDa}$ was located predominantly (93–96%, n = 2) in the nuclear fraction. When compared using equal protein loadings, calbindin$_{28kDa}$ was more evenly distributed among the three particulate fractions (Fig. 1B). To ensure that the apparent insolubility of calbindin$_{28kDa}$ did not reflect incomplete tissue disruption, cerebellum was subjected to three cycles of homogenisation and ultracentrifugation. Analysis of immunoblots and parallel gels stained with Coomassie blue showed that the specific abundance of calbindin$_{28kDa}$ after three homogenisation steps was 90% (n = 2) compared to that after a single step (see

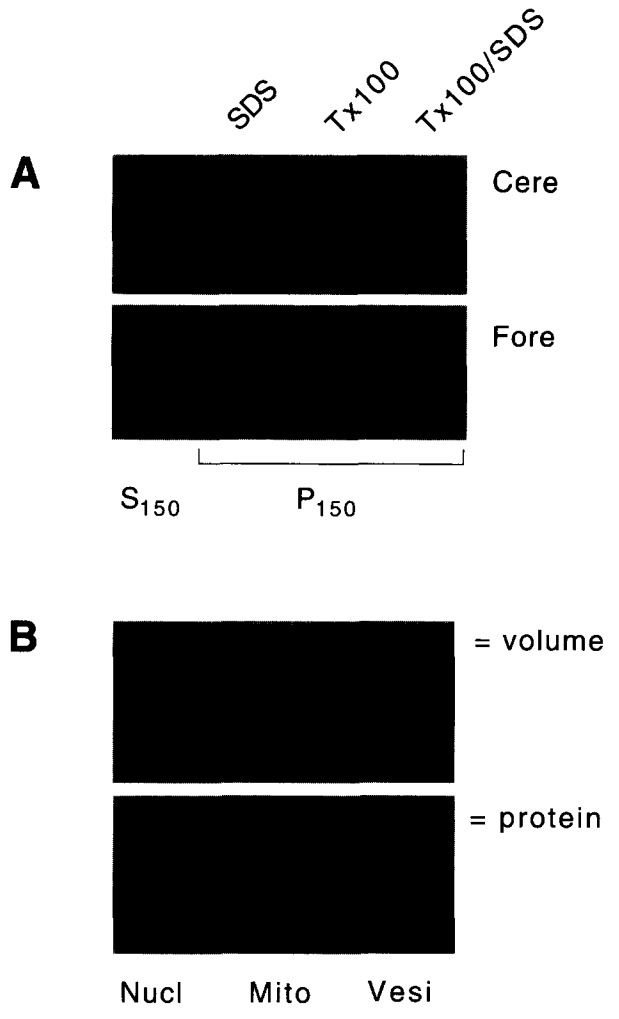

Fig. 1. Subcellular localisation of calbindin$_{28kDa}$ in rat brain by immunoblotting. Homogenates of cerebellum (Cere) and forebrain (Fore) were ultracentrifuged to give the soluble (S_{150}) and total particulate (P_{150}) fractions, or differentially centrifuged to give the crude nuclear (Nucl), mitochondrial (Mito) and microvesicular (Vesi) fractions, as described in section 2. No immunoreaction product accumulated outside the regions shown, as illustrated elsewhere [31]. (A) The once-washed pellets were extracted with buffer (10 mM Tris-HCl, pH 7.2, 2 mM dithiothreitol) containing 2% SDS (at 100°C) or 2% Triton X-100 (at 4°C) as indicated. In lane 4, the Triton-insoluble residue from lane 3 was extracted with 2% SDS. All fractions were adjusted to the original volume of homogenate before 1 μl samples were taken. (B) The pellets from cerebellum were extracted with SDS and sampled to give equal volume or equal protein loads (based on Coomassie blue-stained SDS-PAGE), as indicated. Cerebrum gave the same result.

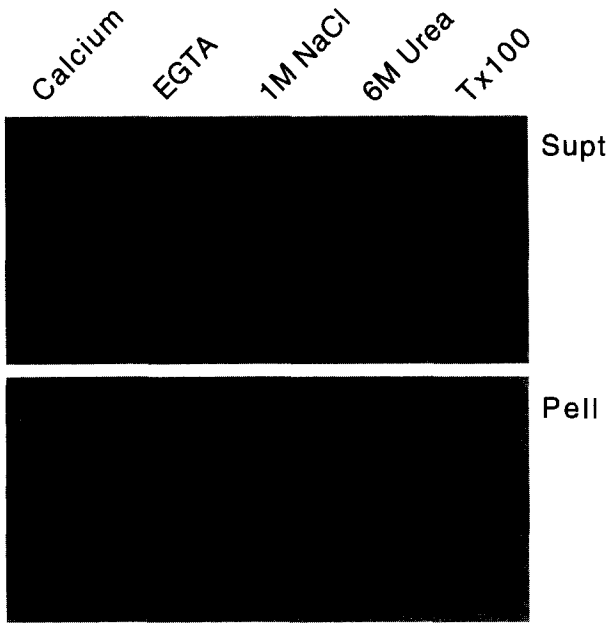

Fig. 2. Solubility analysis of particulate calbindin$_{28kDa}$. Samples of total particulate fraction from whole brain were resuspended for 30 min in ice-cold homogenisation buffer containing the following additives, then ultracentrifuged and the resultant soluble (Supt) and particulate (Pell) fractions (2 μl samples) immunoblotted: lane 1 = no additive; lane 2 = 10 mM EGTA; lane 3 = 1 M NaCl (final concentration); lane 4 = 6 M urea; lane 5 = 2% Triton-X100.

above), indicating that the standard homogenisation was essentially complete (data not shown). Moreover, this and another experiment with multiple wash steps (Fig. 2) confirmed that the particulate localisation did not reflect trapping of cytosol in the interstices of the pellet.

3.2. Characterisation of particulate calbindin$_{28kDa}$

To assess more stringently the apparent equivalence of soluble and particulate calbindin$_{28kDa}$ (Fig. 1A), samples were subjected to denaturing two-dimensional gel electrophoresis with immunodetection. No differences were detected between Triton-solubilised and cytosolic calbindin$_{28kDa}$, both when analysed individually and as a mixture, indicating identity. Particulate calbindin$_{28kDa}$ also retained the ability to bind calcium, as assessed by ^{45}Ca-overlay analysis (data not shown).

Several calcium-binding proteins, including calmodulin and the annexins, are known to undergo calcium-dependent interactions with components of the particulate fraction. When brain pellets were extracted with the calcium chelator EGTA, only a minimal increase of solubilised calbindin$_{28kDa}$ was obtained compared with the standard buffer that contained ≈10 μM calcium (Fig. 2). While high ionic strength (1 M NaCl) also had little effect, 6 M urea released a significant amount (18–22%, n = 2) of calbindin$_{28kDa}$. Together, these findings and the almost complete solubility in Triton X-100 (Figs. 1A and 2) suggested that calbindin$_{28kDa}$ was tightly bound to, or sequestered within, Triton-labile components of the particulate fraction.

To establish whether particulate calbindin$_{28kDa}$ was located inside or outside organelles, we tested its accessibility to trypsin. Without Triton X-100, only 43 ± 3% (n = 3) of calbindin$_{28kDa}$ was degraded compared to digests in the presence of detergent (Fig. 3), suggesting a dual localisation within and outside membrane-limited compartments. Cytosolic calbindin$_{28kDa}$ was completely degraded within 30 min under these conditions (data not shown).

3.3. Subcellular fractionation and characterisation of calbindin$_{30kDa}$

We considered the possibility that the putative functional differences between calbindin$_{28kDa}$ and calbindin$_{30kDa}$ might manifest as a differential subcellular localisation. The subcellular fractionation pattern of calbindin$_{30kDa}$ in cerebellum and cerebrum (Fig. 4A) was found to parallel that of calbindin$_{28kDa}$ (Fig. 1A), but densitometry revealed that a significantly smaller proportion of calbindin$_{30kDa}$ (27 ± 1% of the total, n = 3; P < 0.02) was particulate. The partitioning of calbindin$_{30kDa}$ between the crude nuclear, mitochondrial and vesicular fractions (Fig. 4B) also mimicked that of calbindin$_{28kDa}$ (Fig. 1B). Likewise, in the other experiments described above, these two calcium-binding proteins showed equivalent behaviour (data not shown).

4. Discussion

This study showed that a substantial proportion of calbindin$_{28kDa}$ and calbindin$_{30kDa}$ (36% and 27% of the total, respectively) is located in the particulate fraction of rat brain, conflicting with the general view that these high affinity calcium-binding proteins are essentially soluble constituents of the cytosol. Partitioning of the calbindins into the particulate fraction did not appear to result from their posttranslational modification and was not modulated by calcium in a reversible 'calmodulin-like' manner. Other evidence pointed to a dual compartmentalisation of particulate calbindins, both within

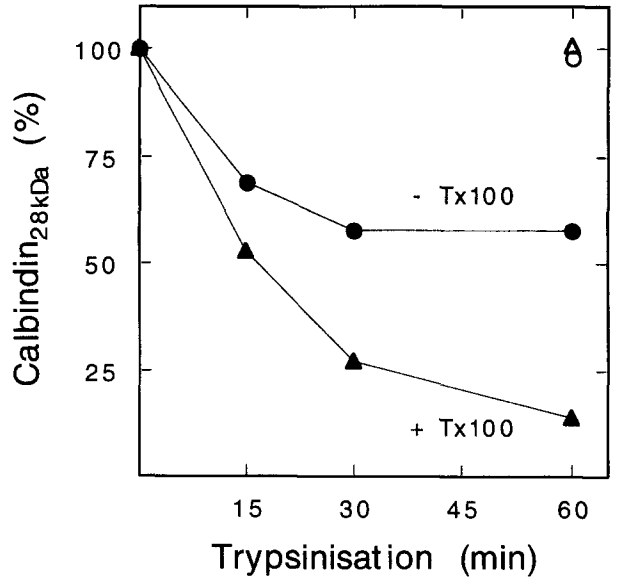

Fig. 3. Accessibility of particulate calbindin$_{28kDa}$ to trypsin. Samples of total particulate fraction from whole brain (prepared minus protease inhibitors) were suspended in 20 μl of 100 mM Tris-HCl, pH 8.0, containing 2% Triton X-100 where indicated. Following incubation at 37°C, with and without 1 μg trypsin (closed and open symbols, respectively), the reactions were stopped at the indicated times by boiling in SDS-PAGE sample buffer then immunoblotted.

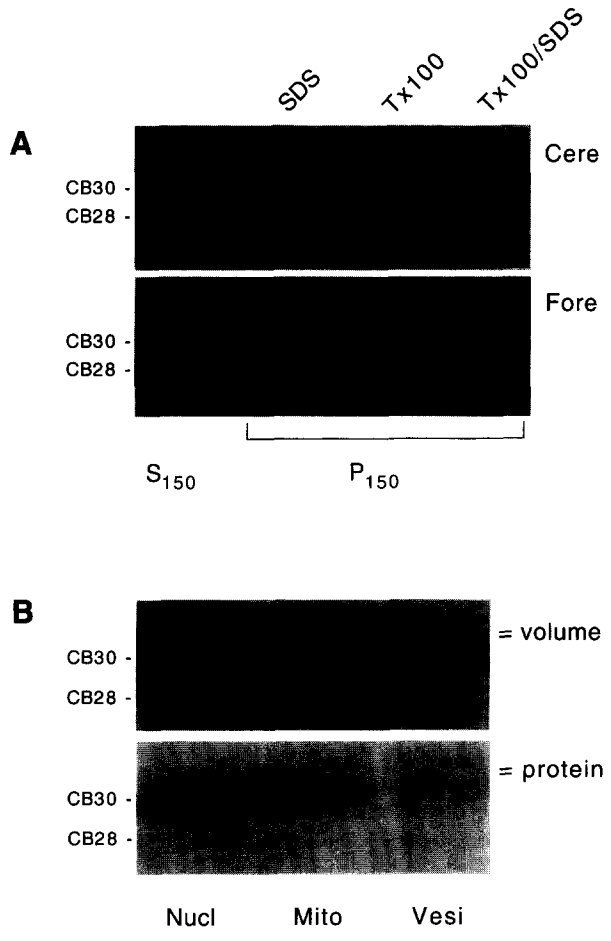

Fig. 4. Subcellular fractionation of calbindin$_{30kDa}$. Samples from the experiment in Fig. 1 were immunoblotted with anti-calbindin$_{30kDa}$. The positions of calbindin$_{30kDa}$ (CB30) and calbindin$_{28kDa}$ (CB28) are indicated.

and outside organelles. Together with similar data from rat enamel cells reported elsewhere [31], these findings suggest that calbindin$_{28kDa}$ and calbindin$_{30kDa}$ have target ligands and major signalling functions in the insoluble cellular fraction. Consequently, a substantial proportion of these calbindins is unlikely to function as mobile buffers or transporters of calcium in the cytosol.

The relatively high proportion of particulate calbindin$_{28kDa}$ detected here in brain, and earlier in enamel cells [31], could result jointly from the detection approach we used and tissue-specific differences in calbindin$_{28kDa}$ compartmentalisation. In combination with SDS-PAGE, immunoblotting enabled the particulate fraction to be extracted harshly (i.e. by boiling in SDS) and the immunoquantitated species to be visualised and validated. Conversely, previous quantitations by radioimmunoassay were done on samples extracted with nonionic detergent or without detergent [23–25]. Given the poor solubilisation of brain calbindin$_{28kDa}$ without Triton X-100 (Fig. 2), the low value reported previously (≈5% insoluble in neostriatum [25]) might simply have reflected the omission of detergent from the extraction procedure. Concordantly, with intestine samples, values obtained for particulate calbindin$_{28kDa}$ without Triton X-100 were two-fold lower than in its presence [23,24]. However, even the Triton values might be underestimates in some tissues since most particulate calbindin$_{28kDa}$ was Triton-insoluble in enamel cells [31]. It will be of interest to apply the immunoblotting approach to other tissues and establish whether the insoluble behaviour observed in brain and enamel cells is a general phenomenon. A preliminary analysis of kidney indicated that particulate calbindin$_{28kDa}$ exceeded 15% of the total (unpublished data). Several potential artefacts associated with subcellular fractionation (i.e. freeze/thawing effects, incomplete tissue disruption or washing of pellet) were addressed here, and others showed that insoluble calbindin$_{28kDa}$ did not result from vesicular entrapment in duodenum extracts [21]. The challenge remains to relate the in vitro findings, obtained with subcellular fractionation and other approaches, to the subcellular localisation of calbindin that exists in vivo.

Our biochemical evidence that much calbindin$_{28kDa}$ is insoluble supports some previous immunocytochemical data, but further investigations will be required to identify the target locations within the particulate fraction and to determine the distribution of calbindin$_{28kDa}$ between them. A consistent finding in this and most earlier subcellular fractionation studies is that insoluble calbindin$_{28kDa}$ partitions mainly with the crude nuclear fraction (Fig. 1B and [23–25,31]; exception is [22]). The partial protection of particulate calbindin$_{28kDa}$ from trypsin (Fig. 3 and [31]) indicates localisation within membrane-limited organelles, such as the nucleus. Several immunocytochemical studies have noted an intranuclear concentration of calbindin$_{28kDa}$ but descriptions of the relative abundance in this location vary widely, even differing between studies of the same tissue [4,23,25,27]. In recent studies of brain and intestine, the predominant localisation of calbindin$_{28kDa}$ was found to be inside vesicular organelles (identified as lysosomes) associated with the cytoskeleton [5,27]. While such a distribution would be consistent with the observed near total solubility in Triton X-100 (Figs. 1A and 2), the accessibility to trypsin indicates that approximately half of the brain particulate calbindin$_{28kDa}$ is extravesicular (Fig. 3), although a contribution from leaky vesicles cannot be ruled out. Together, the solubility and trypsin experiments (Figs. 2 and 3) imply that extravesicular calbindin$_{28kDa}$ is tightly associated, in a calcium-independent manner, with Triton-labile particulate structures. These characteristics point to the existence of high affinity target ligands for calbindin$_{28kDa}$, perhaps associated with the outside of organelles or with the cytoskeletal components that fractionate into the crude nuclear pellet. Given the marked differences in solubility (i.e. in Triton X-100 and urea) between brain calbindin$_{28kDa}$ (Fig. 2) and that from enamel cells [31], it seems likely that different target ligands predominate in various tissues. Further investigations are warranted to identify the putative ligands and to elucidate the nature and function of their interaction with calbindin$_{28kDa}$. It is noteworthy in this regard that we found soluble and particulate calbindin$_{28kDa}$ to be indistinguishable by two-dimensional immunoblotting, implying that interaction with the targets does not arise from posttranslational modification (e.g. phosphorylation) of calbindin$_{28kDa}$.

Two principal functional implications arise from the observed insolubility of calbindin$_{28kDa}$. First, the novel evidence for target ligands, noted above, supports the idea that calbindin$_{28kDa}$ has important functions other than binding calcium, perhaps by being part of a stable protein complex. The putative target proteins seem to be either absent from the cytosol or

present in a nonbinding state, since cytosolic calbindin$_{28kDa}$ behaves as a monomer during size exclusion chromatography [21,31]. The evidence that the binding of calbindin$_{28kDa}$ is calcium independent, not being substantially reversed by EGTA after 30 min (Fig. 2), precludes a classical calmodulin-like interaction. However, it is pertinent that in some cases calmodulin binding is not readily disrupted by EGTA [34] and that some calmodulin-like proteins (e.g. the B subunit of calcineurin) form tight complexes with other proteins (i.e. the catalytic subunit, calcineurin A) which persist in the presence of EGTA [35]. Hence it now seems reasonable to propose that calbindin$_{28kDa}$ could be the calcium-signalling component of a multisubunit protein. Second, our observation in brain and enamel cells, that a major proportion of calbindin$_{28kDa}$ is effectively immobile in the insoluble fraction, is at variance with its putative roles as a diffusible cytosolic protein involved in calcioprotective buffering or calcium transport. While intravesicular calbindin$_{28kDa}$ could possibly be involved in calcium transcytosis through bulk vesicle translocation [5], the quantitative basis of this proposed mechanism is open to question [3]. Further work will be required to establish whether the soluble pool of calbindin$_{28kDa}$ has a separate function, centred on calcium buffering, or instead acts as a reservoir for the proposed signalling-based insoluble pool.

Finally, the finding that calbindin$_{30kDa}$ behaves like calbindin$_{28kDa}$ during subcellular fractionation supports the notion that calbindin$_{30kDa}$ too has target ligands, as suggested by its strikingly conserved primary structure [28]. The decreased proportion of insoluble calbindin$_{30kDa}$, relative to calbindin$_{28kDa}$ (Figs. 1A and 4A), is consistent with these cognate proteins having similar but nonidentical functions.

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