Expression of a Neuronal Ca\(^{2+}\)/Calmodulin-dependent Protein Kinase, CaM Kinase-Gr, in Rat Thymus*

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The regional and tissue-specific expression of the Ca\(^{2+}\)/calmodulin-dependent protein kinase, CaM kinase-Gr, were examined. The M, 65,000 α-polypeptide of CaM kinase-Gr is expressed ubiquitously in different anatomical regions of rat brain, whereas an additional M, 67,000 β-polypeptide is observed solely in the cerebellum. The α-polypeptide appears in the neonatal rat forebrain and cerebellum, whereas the β-polypeptide appears by the second postnatal week and may reflect cerebellar granule cell differentiation. Most peripheral tissues do not express either CaM kinase-Gr polypeptide. However, rat thymus and thymocytes derived therefrom express CaM kinase-Gr at levels comparable to those of the central nervous system. The identity of the enzyme in rat thymus was corroborated by immunoblot assays, Northern blots, and direct enzyme purification. Rat spleen and testis also produce CaM kinase-Gr, but at lower levels than either thymus or brain. These observations demonstrate selective regional and developmental expression of CaM kinase-Gr polypeptide in brain, and suggest that it may participate in Ca\(^{2+}\) signalling in cells derived both from the immune system as well as the central nervous system.

Ca\(^{2+}\)/calmodulin-dependent protein kinase type "Gr" was initially described as an abundant neuronal protein kinase which was particularly enriched in rat cerebellar granule cells, but was also present in other neuronal subtypes (1). Screening mouse (2) and human (3) brain cDNA libraries resulted in partial nucleotide sequences of cDNA species which appear to code for homologues to the rat enzyme. Along with cyclic AMP-dependent protein kinase, CaM kinase-I (1), and protein kinase C, CaM kinase-Gr appears to represent a "multifunctional" protein kinase which is regulated by a second messenger.

CaM kinase-Gr can be purified as two monomeric polypeptides with M, values of 65,000 and 67,000 (1) which, by analogy with CaM kinase-II (4), are comprised of catalytic, regulatory, and association domains (5). A testis-specific calmodulin-binding protein, calsperrin (6), was found to be identical to a combination of the regulatory and association domains in the absence of the catalytic domain (1, 5, 6). The gene-encoding for CaM kinase-Gr proved to contain within it a gene coding for calsperrin whose transcription appears to be under the control of a separate promoter (5). In addition to differential transcription initiation, differential splicing, and alternative polyadenylation reactions lead to the generation of distinct mRNAs for CaM kinase-Gr and calsperrin (5).

CaM kinase-Gr occurs both in soluble and nuclear subcellular compartments (7, 8) where it could mediate different aspects of Ca\(^{2+}\) signalling. It phosphorylates proteins which are substrates for either CaM kinase-II or cyclic AMP-dependent protein kinase with a preference for the former; two of these substrates are synapsin I (1) and raplb (9). The present study was undertaken to investigate the regional distribution of the M, 65,000–67,000 enzyme polypeptides in rat brain, and their possible occurrence in peripheral tissues. Based on the observations reported here, it appears that CaM kinase-Gr expression achieves its highest levels in thymus as well as brain where it is expressed by thymic lymphocytes and neurons, respectively. Lower levels of expression were observed in spleen and testis, but in no other tissue that was examined. The presence of relatively high levels of CaM kinase-Gr in lymphocytes as well as neurons suggests that it may be a target for Ca\(^{2+}\) action in the immune system as well as the central nervous system.

MATERIALS AND METHODS

\([γ-\text{32P}]\text{ATP} (\sim 30 \text{ Ci/mm})\] was purchased from Du Pont-New England Nuclear, and syntide-2 was synthesized by J. McDermed, Wellcome Research Labs; calmodulin and calmodulin-agarose were from Sigma and Ficoll-Paque from Pharmacia. Rabbit antiserum against a rat brain recombinant CaM kinase-Gr-gal fusion protein was raised as described previously, and monospecific antibody preparations were obtained by affinity purification using purified, native rat brain enzyme (1). Suitable dilutions for immunoblot experiments were determined to be 1:500 for the antisera and 1:100 or 1:50 for the affinity-purified immunoglobulin. The specificity of the α/β polypeptide immunoreactivity of CaM kinase-Gr was confirmed throughout by deleting the primary antibody, or neutralizing it with native brain enzyme, or by the use of affinity purified, monospecific antibody preparations.

Various tissues were obtained from Sprague-Dawley rats or Charles Rivers CD-1 mice and homogenized in 6 volumes of 25 HEPEs, pH 7.5, 2 mM EDTA, 2 mM EGTA, 2 mM dithiothreitol, 20 mM sodium pyrophosphate, 10 μg/ml leupeptin, and 100 μg/ml phenylmethylsulfonyl fluoride. The 250,000 × g supernatants, where the majority of CaM kinase-Gr was localized, were used for immunoblot analysis. The enzyme was purified from rat brain and thymus as described previously by ammonium sulfate precipitation and chromatography on DEAE-cellulose, AcA-34, and calmodulin-agarose columns (1). Protein concentrations were measured by the Bradford method (10), and polypeptides were resolved by SDS-PAGE using 8% acrylamide (11); electrophoretic transfer to nitrocellulose sheets and immunoblot analysis were carried out as detailed previously (1). Thymocytes and
splenocytes were obtained by subcapsular perfusion with Dulbecco’s modified Eagle’s medium. Contaminating red cells in the splenocyte preparations were removed with NH4Cl lysis buffer, and thymocytes were further purified by centrifugation over Ficoll-Paque. Cells were lysed by sonication in 6 volumes of a buffer containing 50 mM HEPES, pH 7.5, 0.1% Triton X-100, 4 mM EGTA, 15 mM EDTA, 20 mM sodium pyrophosphate, 100 µg/ml phenylmethylsulfonyl fluoride, and 10 µg/ml leupeptin, and the soluble fraction was obtained after Microfuge centrifugation. CaM kinase-Gr activity was measured in a 30-µl reaction mixture containing 5–10 ng of enzyme, 20 µM syntide-2, 50 µM ATP, 1–2 µCi of [γ-32P]ATP, 10 mM MgCl2 with or without 5 mM CaCl2 and 600 nM calmodulin; the assays were performed for 6 min at 25°C and 3P incorporation was measured by the P-81 filter paper method (12).

RNA was prepared from different tissues, poly(A)-selected and probed with radiolabeled c-36 cDNA which codes for the C-terminal HEPES, HPLC method (12).

Results

Various regions of the rat brain were isolated and examined for the presence of the M, 65,000 and 67,000 polypeptides which will be referred to as the α- and β-polypeptides, respectively (Fig. 1A). Cytosolic extracts from the different brain areas displayed CaM kinase-Gr α-polypeptide immunoreactivity with higher levels in the cerebellum, striatum, frontal cortex, and amygdala, lower levels in the thalamus, hypothalamus, and brain stem, and intermediate levels in the hippocampus. The expression of the β-polypeptide seemed to be confined to the cerebellum and is likely to derive from the granule cells therein (1, 7, 8). We had previously demonstrated by immunohistochemical examination that CaM kinase-Gr is produced by specific neuronal subtypes starting during the embryonic and neonatal periods (8). Here we show that the α-polypeptide is indeed expressed both in neonatal forebrain and cerebellum, but that its levels increase markedly during postnatal week only. Whether this transiently appearing form of the enzyme is identical to the cerebellar β-polypeptide is unclear.

Most rat organs including liver, lung, kidney, heart, and skeletal muscle did not manifest α/β-polypeptide immunoreactivity which could be detected at the level of sensitivity of the immunoblot assay (not shown). However, both spleen and testis displayed modest levels of α-polypeptide immunoreactivity (Fig. 2A). The presence of CaM kinase-Gr immunoreactivity in the spleen prompted us to inquire further into the possible generation of the kinase in elements of the immune system. Consequently, rat thymus extracts were analyzed for their content of CaM kinase-Gr immunoreactive polypeptides (Fig. 2B). Surprisingly, intense immunoreactivity which comigrated with the α-polypeptide was observed in soluble thymic extracts. Both the molecular nature and cellular origin of this immunoreactivity were explored. Thymocytes were isolated from rat thymus and shown to retain the same level of immunoreactive protein as the intact thymus.

Ca2+ Signaling in Neurons and Lymphocytes
CaM signaling in Neurons and Lymphocytes

(Fig. 2C) implying that these cells indeed express high levels of CaM kinase-Gr. Likewise, isolated splenocytes retained comparable immunoreactivity to the intact spleen (not shown). Interestingly, thymus and thymocytes consistently exhibited faint immunoreactivity slightly above the $\alpha$-polypeptide following immunoblot analysis (Fig. 2, B and C). Whether this represents another form of the enzyme with intermediate electrophoretic mobility between the $\alpha$- and $\beta$-polypeptides is unclear. This putative intermediate form of the enzyme was, however, not observed after enzyme purification (see Fig. 4).

Not only did spleen and thymus express CaM kinase-Gr immunoreactive protein, but they also contained two major mRNA species at 2.3 and 3.5 kb (Fig. 3) which hybridized to a CaM kinase-Gr cDNA probe, and had approximately the same size as the corresponding mRNA species (2.1 and 3.5 kb) previously seen in rat forebrain and cerebellum (1). We had postulated that a 2.1-kb mRNA species codes for the $M_i$ 65,000 ($\alpha$)-polypeptide of CaM kinase-Gr, and that the 3.5-kb mRNA utilizes a different polyadenylation site further downstream from that of the 2.1-kb species (5). The intensity of the hybridization signal for the 2.3-kb mRNA observed here is considerably greater in thymus than in spleen, in agreement with the levels of CaM kinase-Gr immunoreactivity in these two organs. In contrast, the levels of the 3.5-kb mRNA appear to be similar in spleen and thymus, and the precise role of this mRNA in the generation of CaM kinase-Gr remains to be elucidated. Moreover, several minor mRNA components which hybridize with the cDNA probe were consistently observed in brain and thymus; their significance is currently under investigation.

The relatively high levels of enzyme in rat thymus permitted its direct isolation following the same experimental protocol originally devised (1) to purify the enzyme from rat brain (Fig. 4). The purified enzyme preparation contained one major $M_i$ 65,000 polypeptide, both by silver staining and immunoblotting (Fig. 4A), which represents the $\alpha$-subunit of CaM kinase-Gr. As expected, the purified thymic enzyme was able to phosphorylate the synthetic peptide substrate, syntide-2, in a Ca$^{2+}$/calmodulin-dependent reaction (Fig. 4B). Some enzyme preparations displayed partial stimulation in the presence of Ca$^{2+}$ without calmodulin (not shown). Whether these preparations were contaminated with another Ca$^{2+}$-sensitive kinase or kinase activator or whether the Ca$^{2+}$ response derived from CaM kinase-Gr itself remains to be elucidated.

The above data collectively provide evidence for the expression of CaM kinase-Gr in rat thymus and spleen. However, differences between the neuronal enzyme and that of thymocytes and splenocytes remain possible, but not detectable by the methods used in this study. Potential differences include variations in the amino acid sequence which result from tissue-specific alternative RNA splicing, and posttranslational modification. Moreover, previous cDNA cloning and sequencing studies and chromosomal localization had suggested the existence of mouse and human homologues of CaM kinase-Gr, although a substantial degree of sequence divergence was observed (1–3). Therefore, we sought to verify the actual presence of mouse and human polypeptides which cross-react with antibodies raised against the rat enzyme. The monospecific antiserum against the rat C-terminal half of CaM kinase-Gr (1) was employed to search for cross-reactive mouse and human proteins. Mouse cerebellum and thymus each contained an immunoreactive polypeptide with a $M_i$ value of 65,000 which probably corresponds to the $\alpha$-polypeptide of the kinase (Fig. 5). The magnitude of immunoreactivity in mouse brain and thymus was comparable to that in rat spleen (Fig. 5), and considerably less than rat brain or thymus. The weaker immunoreactivity of the mouse compared to the rat polypeptides is likely to stem, at least in part, from the amino acid sequence differences between the rat and mouse enzymes, particularly in the antigenic poly Glu region (1), suggesting that antiserum against the rat enzyme may have a lower affinity for the mouse enzyme. A combination of low affinity for the antibody and lesser levels of CaM kinase-Gr may explain our inability to detect the kinase in mouse spleen and testis (not shown) or the $\beta$-polypeptide in mouse brain. An even greater degree of sequence divergence between the human (3) and rat (1, 5) CaM kinase-Gr may account for our failure to detect CaM kinase-Gr in various human lymphocyte cell types using the anti-rat enzyme antiserum (not shown). Resolving this issue requires the identification, purification,

![FIG. 3. Expression of Cam kinase-Gr mRNA in thymus (Th) and spleen (S).](image-url)

![FIG. 4. Purification of rat thymus CaM kinase-Gr.](image-url)
and immunochemical characterization of the presumed human enzyme itself.

**DISCUSSION**

Previous reports (1, 4, 7, 8, 13, 14) and the present data provide evidence that neurons contain two multifunctional Ca\(^{2+}\)/calmodulin-dependent protein kinases, CaM kinase II and CaM kinase-Gr. The ratio of the two enzymes varies as a function of neuronal subtype, stage of development, and polypeptide composition. Thus, neurons can exploit the presence of two multifunctional Ca\(^{2+}\)/calmodulin-signalling pathways which are endowed with different characteristics. These two molecular pathways can potentially confer on neurons both redundancy and versatility in Ca\(^{2+}\) signalling. Ca\(^{2+}\) has also featured prominently as a second messenger involved in lymphocyte development and activation (reviewed in Refs. 15–19). Nevertheless, evidence for the participation of Ca\(^{2+}\)/calmodulin-dependent protein kinases in lymphocyte Ca\(^{2+}\) signalling has not been firmly documented. The enrichment of CaM kinase-Gr in thymocytes raises the possibility that one or more Ca\(^{2+}\)/calmodulin-dependent protein kinase may be a target for Ca\(^{2+}\) signalling in lymphocytes in general, and T-cells in particular. A variety of stimuli can lead to increased transient increase in lymphocyte Ca\(^{2+}\) levels, whereas in non-T-cells Ca\(^{2+}\) is not easily mobilized. The generation of IP\(_3\) may be the required in addition to Ca\(^{2+}\) to achieve T-lymphocyte activation (15, 18, 22). Ca\(^{2+}\) itself induces protein kinase C to associate with membranes and potentiate the effects of diacylglycerol/phorbol esters (23, 24); however, as in other cell types, in lymphocytes it is likely that Ca\(^{2+}\) acts on multiple molecular targets. Ca\(^{2+}\)/calmodulin-dependent protein kinases would represent one such potential target. The identification of suitable Ca\(^{2+}\)/calmodulin-dependent protein kinases in lymphocytes which would mediate the effects of Ca\(^{2+}\) on lymphocyte activation has been somewhat elusive. CaM kinase-II has been identified by immunoblots of calmodulin-binding proteins from spleen (25), and CaM kinase-I activity has been measured in spleen cytosolic extracts using phosphorylation of synapsin I on site I as an assay (26). Nevertheless, determining the actual presence and levels of these two protein kinases in lymphocytes may require further investigation.

The presence of CaM kinase-Gr in thymocytes at levels comparable to those of its richest source, rat cerebellum, is compatible with a possible role for this enzyme in T-lymphocyte development and activation. The potential function of CaM kinase-Gr in T-lymphocyte Ca\(^{2+}\) signalling would partly depend on its subcellular localization and possible association with nuclear chromatin (7), response to specific extracellular ligands, and lymphocyte subtype and stage of differentiation. A search for CaM kinase-Gr substrates in lymphocytes would also be essential in elucidating its function. Candidate substrates for a lymphocyte Ca\(^{2+}\)/calmodulin-dependent protein kinase possessing the catalytic specificity of CaM kinase-Gr include the nuclear proteins encoded by c-ets-1 and c-ets-2 (27, 28) and the cyclic AMP/Ca\(^{2+}\)-regulated CI- channel which has been implicated in the pathogenesis of cystic fibrosis (29).

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