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Transglutaminase Forms Midkine Homodimers in Cerebellar Neurons and Modulates the Neurite-Outgrowth Response

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Midkine is a prominent acyl donor substrate for the protein cross-linking enzyme transglutaminase type 2 in rat brain neurons. Transglutaminase type 2 and midkine immunoreactivity are regionally colocalized in developing cerebellar cortex. Monomeric midkine is present in the embryonic dorsal rhombic lip which gives rise to the cerebellar cortex. A high-molecular weight (29-30 kDa) midkine appears during postnatal cerebellar development. The presence of the high-molecular weight midkine in cultured cerebellar cortical interneurons is dependent upon culture conditions. Transglutaminase catalyzes the calcium-dependent cross-linking of midkine predominantly into 29-30 kDa dimers. Dimer-formation of midkine *in vitro* and in cultured neurons is reduced in the presence of a transglutaminase inactivator. Neurons plated onto previously cross-linked midkine exhibit larger growth cones and enhanced neurite outgrowth compared to those plated onto monomeric midkine alone.

Midkine is a highly basic heparin-binding cytokine (1) which is expressed transitionally in the mid-gestation mammalian embryo in kidney, brain and epithelial tissues and in regions of prominent secondary induction (2). In developing brain midkine becomes associated predominantly with the external surface of neural cell membranes (3). The peptide was first discovered as the product of a gene whose expression was stimulated by retinoic acid in embryonal carcinoma cells (1). It was subsequently shown to have potent biological activities in promoting neurite-sprouting in nerve cells (4,5) and as a developmental morphogen (6). Although the structure, pattern of expression and biological activities of midkine have been well-documented (7), its mode of action and putative receptor signalling pathways are not known.

In a number of tissues, highly stable dimeric and trimeric forms of midkine have been reported (8,9). In view of the non-reducible nature of these multimeric complexes, it has been proposed that, in the context of receptor activation, midkine can participate in intermolecular interactions through non-disulphide covalent cross-linking. In cultured cerebellar neurons, we have previously demonstrated that cell surface-associated midkine is a substrate for transglutaminase at axonal adhesion complexes (10).

Midkine is amongst a growing number of structural proteins, proteases and protease inhibitors associated with the cell surface (including galectin-3 (11), fibrillar collagen type V/XI (12), fibronectin/osteopontin (13), fibronectin/plasminogen (14) and elafin (15)) which undergo homo- or heteropolymer-formation mediated by transglutaminase type 2. In view of the appar-

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Abbreviations: drg, dorsal root ganglion; drl, dorsal rhombic lip; GFRM, growth factor-reduced matrigel; HBNF, heparin-binding neurotrophic factor; hrMK, human recombinant midkine; PLO, poly-L-ornithine; SDS–PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis.

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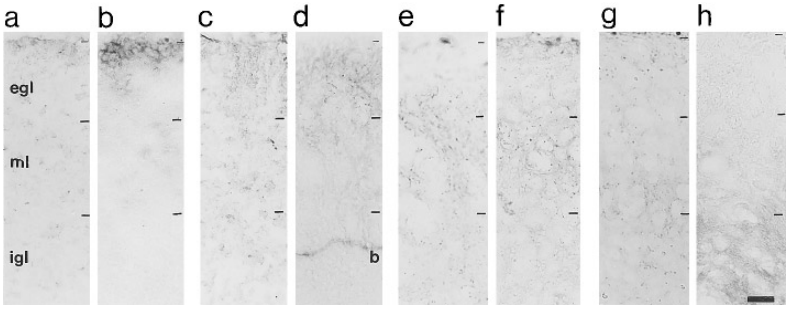

FIG. 1. Immunohistochemical localization of transglutaminase type 2 (a,c,e,g) and midkine (b,d,f,h) in rat cerebellar cortical sections (15 μm) at E19 (a,b) and 3 (c,d), 7 (e,f), and 12 (g,h) days *post partum.* Cerebellar cortical laminae are indicated: egl—external granular layer, ml—molecular layer, igl—internal granular layer. Midkine immunoreactivity was observed in blood vessels (b) in some sections. Calibration bar, 50 μm.

ently irreversible nature and remarkable stability of covalent complexes catalyzed by transglutaminases, the possible implications of such a biotransformation for physiological processes at cellular level are far-reaching. In this study we provide further evidence that the formation of midkine complexes which are predominantly dimeric in rat cerebellar cortex during postnatal development may be catalyzed by transglutaminase type 2. We also demonstrate the modulation by cross-linking of the neurite outgrowth response to midkine which is relevant to this period of cerebellar development.

MATERIALS AND METHODS

Materials. Purified human recombinant MK (hrMK) was prepared as previously described (16). Guinea-pig liver (type 2) transglutaminase was obtained from Sigma. Reagents for immunocytochemical studies and Western blotting were obtained from Sigma and Vectastain. Rabbit antiserum to hrMK conjugated to Keyhole Limpet Hemocyanin was prepared by Berkeley Antibody Co., Richmond, CA. Rabbit antiserum to bovine transglutaminase type 2 was the gift of Dr. S. Hirose.

Tissue preparation and Western Blotting. Immunocytochemistry on cryostat sections was as previously described (10). Tissue extracts were made from embryonic rat rhombencephalic dorsal lip and foetal and postnatal rat cerebellum by glass/glass homogenization then sonication in 0.05 M tris HCl (pH 7.5), 0.25 M sucrose, 1 mM EDTA, 0.1 mM phenylmethylsulfonylfluoride, 20 mM benzamidine and 0.1 mM chymostatin at 0.1 ml/40 mg wet weight of tissue. Samples were separated by SDS-PAGE, electroblotted to a polyvinylidene difluoride membrane and developed using rabbit anti-midkine with biotin/streptavidin/alkaline phosphatase as the detection system.

Cell culture. Cultures of 7-day-old rat cerebellum were prepared and grown on poly-L-ornithine (PLO) or Growth Factor-Reduced Matrigel (GFRM, Becton-Dickinson)-treated surfaces as previously described (10). Cultures of embryonic (E14) rat dorsal root ganglion (drg) cells were grown on poly-L-lysine-treated surfaces in the absence of Nerve Growth Factor and scored for neurite outgrowth as described (17).

RESULTS AND DISCUSSION

Transglutaminase type 2 immunoreactivity in cerebellar cortex is localized to axonal processes known to be associated for the most part with granule neurons (10). During the period in which granule neurone migration takes place, transglutaminase and midkine were seen to spread through the external granular layer between E19 and day 7 *post partum* (Fig 1a-f). At 12 days post partum, transglutaminase immunoreactivity was less well localized and distributed throughout the molecular layer, whilst midkine was absent from the external granular layer but concentrated in the molecular layer and internal granular layer (Fig 1g-h). Midkine had a similar but not overlapping distribution with transglutaminase type 2 at cellular level. These observations support previous evidence for the expression of both type 2 transglutaminase and midkine in developing rat cerebellar cortex (10, 18) and the possibility that the proteins are synthesized in different cell types (19).

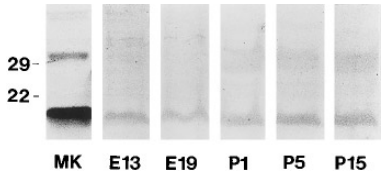

FIG. 2. Presence of monomeric and dimeric midkine revealed by SDS−PAGE with Western blotting during cerebellar ontogeny. Ages indicated in embryonic days' gestation (E) and *post partum* (P). E13 is drl (cerebellar cortex precursor), E19 and thereafter are cerebellum.

Midkine has a molecular mass of 13 kDa and appears as 18 kDa on SDS-PAGE due to its high content of basic amino-acid residues. During the ontogeny of the cerebellar cortex, monomeric midkine was detectable at 13 days' gestation (E13) within the dorsal rhombic lip (drl, precursor to cerebellar cortex) and in the cerebellum at E19 (Fig. 2). However by the time of birth a second immunoreactive protein of apparent molecular weight 29-30 kDa. was also observed. In cerebellar cultures containing both neurones and glial cells grown upon a biological substratum (GFRM), both monomeric and dimeric midkine were present (Fig. 3). In cultures grown on PLO, monomeric midkine alone was present, but conversion to dimers could be induced by the combined action of retinoic acid and dibutyryl cyclic AMP. In the presence of the haloisoxazole active site-directed transglutaminase inhibitor RS-48373-007 (3 μM), the proportion of monomer to dimer was greater than in control cultures, suggesting that transglutaminase inhibition can reduce the conversion of monomer to dimer. The stereoisomer of this compound, RS26752-007, which gives almost no detectable inhibition of transglutaminase C *in vitro* at the same concentration (10), did not suppress dimer-formation.

In order to obtain evidence that high molecular weight midkine is a homopolymer, and not a heteropolymer of midkine with one or more unidentified small proteins in neural tissue or cell extracts, we investigated the formation of complexes from hrMK in the presence of transglutaminase type 2 (Fig. 4). In the presence but not in the absence of Ca^{2+}, transglutaminase type 2 catalyzed the formation predominantly of dimeric midkine, with smaller amounts of trimeric and multimeric complexes. The transglutaminase preparation was found to contain about 5 % of protein impurities, all of which had molecular masses of more than 50 kDa. Thus the complexes observed towards the top of the gel (Fig. 4, lane 6) may contain heteropolymers of midkine with transglutaminase itself and other unidentified proteins. The formation of dimeric midkine was suppressed by exposing cultures to RS48373-007 (3μM).

We investigated the possible biological relevance of covalent dimer-formation in midkine

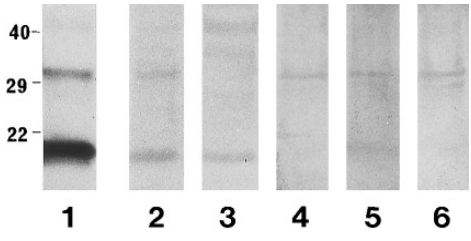

FIG. 3. Influence of culture conditions on conversion of midkine monomer to dimer in cerebellar cortical cultures after 4 days *in vitro* revealed by SDS−PAGE with Western blotting. Lane 1—mixture of monomeric and dimeric hrMK, 2—monomeric and dimeric midkine in culture grown on GFRM. 3—Culture grown on PLO, 4— as for 3 but with inclusion of 10 μM retinoic acid and 50 μM dibutyryl cyclic AMP 24 h before separation, 5— as for 4 but with inclusion of RS48373-007 (3 μM) on day 2 of culture, 6— as for 4 but with inclusion of RS26752-007 (3 μM) on day 2 of culture.

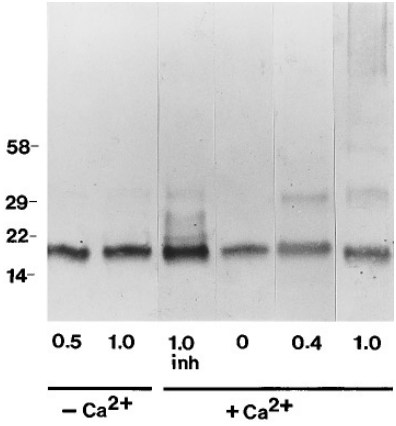

FIG. 4. Demonstration of *in vitro* cross-linking of midkine by transglutaminase type 2 at 37°C in Western blots of reaction mixture separated by SDS–PAGE. hrMK (14μg ml^{-1}) was exposed to transglutaminase type 2 (1 mU) in the presence and absence of Ca^{2+} (1mM). Periods of incubation are shown in h. Inh—presence of RS48373-007 (3 μM). Marker positions (kDa) shown to left.

to its neurite-promoting activity in cultured drg neurons which have growth cones larger than those of granule neurons, making them more suitable for study. Drg cells were grown on a surface coated with PLO followed by hrMK, transglutaminase type 2 or a mixture of hrMK and transglutaminase following conditions for dimer-formation employed as in Fig. 3. Growth cones of axons in cultures seeded for 24h onto cross-linked hrMK were broader and possessed more filopodia than those cultured either on monomeric hrMK or transglutaminase alone (Fig. 5A). In a neurite outgrowth bioassay (Fig. 5B) we found that the precoating of wells with hrMK at concentrations of 1 and 10 ng mg^{-1} could support neurite outgrowth in the absence of nerve growth factor. Covalent cross-linking potentiated the neurite outgrowth response by 1.5-3.0-fold depending on the hrMK concentration.

Midkine with Heparin-Binding Neurotrophic Factor (HBNF)/pleiotropin (20) form a small family of heparin-binding growth factors which are expressed in developing brain and possess potent neurite outgrowth-promoting activity. In the primary sequence of midkine, neurite-promoting activity and proteoglycan-binding moieties are in the C-terminal region (21). Our previous studies identified monomeric midkine as a naturally-occurring acyl donor (glutamine-containing) substrate for the enzyme transglutaminase type 2 at the surface of cultured cerebellar granule neurons (10). HBNF is also a good *in vitro* substrate for transglutaminase type 2 (data not shown), and thus sequences conserved in both peptides which contain glutamine residues, such as $gln^{42/44}$ and gln^{92} for midkine, might serve as transglutaminase substrate domains. Our preliminary data from substrate competition studies with synthetic oligopeptides suggest that gln^{92} is important in dimer-formation. The existence of multiple transglutaminase-mediated polymers containing midkine at the surface of endothelial cells (9) suggests that more than one glutamine or lysine-containing moiety may participate in cross-linking.

Transglutaminase expression resulting in cell-surface cross-linking activity has previously been shown to influence cell behaviour (22), which may be in part due to the modulation of growth factor function (23). Covalent complexes formed by transglutaminases are rendered relatively resistant to proteolysis. It is therefore possible that midkine multimer-formation represents a mechanism for stabilizing the protein and so amplifying its neurite-promoting activity. Part of this mechanism appears to be a direct or indirect effect upon the size and structure of neuronal growth cones. It is also possible that the qualitative pattern of signalling is changed through the covalent polymerization of midkine. Transglutaminase-mediated dimers

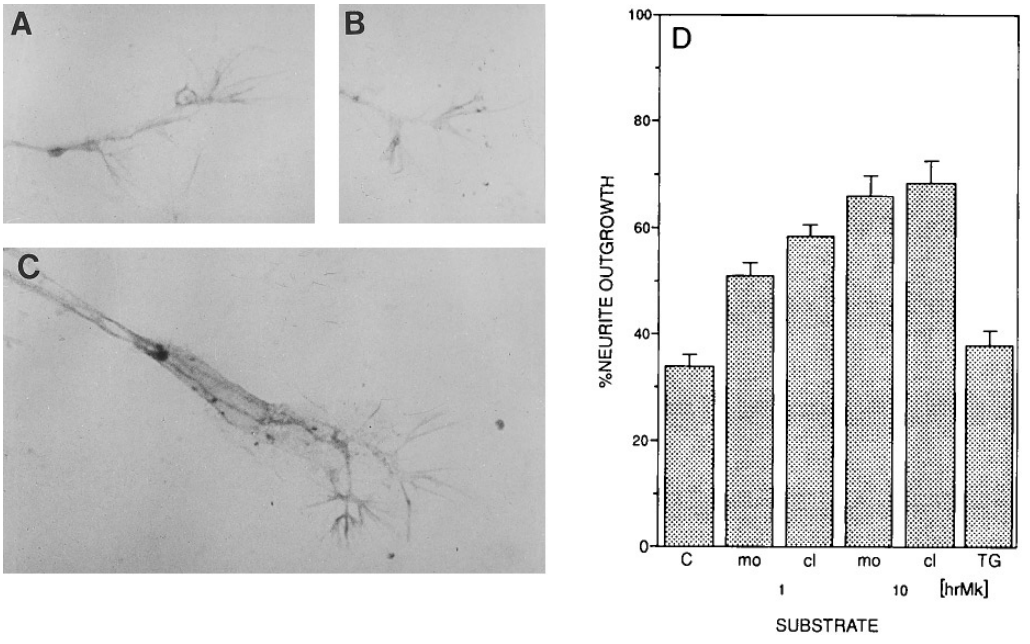

FIG. 5. Influence of midkine cross-linking on growth cone structure and neurite outgrowth response. (A–C) Dorsal root ganglion cell growth cone structure visualized with F-actin immunocytochemistry after 24 h culture on a surface of (A) monomeric hrMK, (B) transglutaminase type 2, and (C) hrMK cross-linked by exposure to transglutaminase type 2 for 4 h, conditions as shown in Fig. 3. (D) Potentiation of neurite outgrowth on monomeric (m) and cross-linked (c) hrMK. Neurite outgrowth score is mean±SEM (n=8). Crosslinking potentiates response to 1 ng ml^{-1} hrMK by 3.0-fold (p=0.005) and to 10 ng ml^{-1} hrMK by 1.5-fold (p=0.023, t-test)

of interleukin-2, unlike monomers, induce apoptosis in oligodendrocytes (24). Furthermore the neurokinin receptor specificity of the neuropeptide substance P is altered by transglutaminase-mediated cross-linking to spermine (25). The formation of growth factor multimers by covalent cross-linking is a novel form of posttranslational modification. Its relevance to signal mechanisms in the short and long-term remain to be studied in detail.

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