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Inositol-1,3,4,5-tetrakisphosphate Binding Sites in Control and *ras*-Transformed NIH/3T3 Fibroblasts

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Inositol-1,3,4,5-tetrakisphosphate (Ins(1,3,4,5)P_4) binding properties were investigated in NIH/3T3 fibroblasts and its *ras*-transformant (DT cells), in which inositol tetrakisphosphates induce Ca^{2+} influx. [^{3}H]-Ins(1,3,4,5)P_4 bound to membranes of both types of cells with K*d* values of 10.6 and 8.6 nM, respectively. The rank order of inositol polyphosphates for displacing [^{3}H]Ins(1,3,4,5)P_4 in DT cells was Ins(1,3,4,5)P_4 > inositol-1,3,4,5,6-pentakisphosphate > inositol hexakisphosphate > inositol-1,4,5-trisphosphate. This order is similar to that reported in two Ras-GTPase-activating proteins, GAP1^{IP4BP} and GAP1^m, which are also the Ins(1,3,4,5)P_4 binding proteins. Northern blot analysis revealed that NIH/3T3 and DT cells expressed mRNA species that were hybridizable with GAP1^m cDNA. These results suggest that parental and *ras*-transformed NIH/3T3 fibroblasts possess GAP1-like proteins, which may be responsible for triggering inositol tetrakisphosphate-dependent Ca^2 influx. © 1997 Academic Press

Stimulation of hormone- and neurotransmitter-receptors activates phospholipid metabolism, and produces inositol-1,4,5-trisphosphate (Ins(1,4,5)P_3) and inositol-1,3,4,5-tetrakisphosphate (Ins(1,3,4,5)P_4) (1-3). These two inositol polyphosphates function to increase intracellular Ca^{2+} concentrations via different routes, which are dependent and independent upon extracellular Ca^{2+} (1,2). Ins(1,4,5)P_3 induces Ca^{2+} mobilization from intracellular Ca^{2+} pools (1,2), whereas Ins(1,3,4,5)P_4 triggers Ca^{2+} influx as observed in lacrimal acinar cells (4), sea urchin eggs (5), and fibroblasts (3,6-8). Recently, it has been reported that Ca^{2+} influx induced by intracellularly-injected Ins(1,3,4,5)P_4 mimics the effect of bradykinin in NIH/3T3 fibroblasts (3,6). This influx becomes prominent in *ras*-transformed NIH/3T3 fibroblast (DT) cells (6,7), in which bradykinin- and Ins(1,3,4,5)P_4-induced Ca^{2+} influx are blocked by a tyrosine kinase inhibitor (8). Thus, the Ins(1,3,4,5)P_4-induced Ca^{2+} influx process seems to be controlled by Ras and tyrosine kinase(s) in the downstream of the signal cascade of bradykinin receptors (9).

Several Ins(1,3,4,5)P_4 binding proteins have been purified from rat (10) or mouse (11) cerebellum and porcine platelets (12). By cDNA cloning, Ins(1,3,4,5)P_4 binding proteins have been characterized as synaptotagmin II (11), a clathrin assembly protein (AP-2; 13) and a GTPase-activating protein (GAP1^{IP4BP}; 14), respectively. These proteins have a binding capacity for various inositol polyphosphates with distinct affinity (10-14). Among them, GAP1^{IP4BP} is the only protein that can interact with H-, R-, or K-Ras through GTP hydrolysis (14). Recently, another GTPase-activating protein, GAP1^m, has been shown to be an Ins(1,3,4,5)P_4 binding protein (15).

In order to elucidate the relationship between inositol tetrakisphosphate ($InsP_4$) binding protein(s) and Ca^{2+} influx and to determine which protein is responsible for the Ca^{2+} influx process, we characterized Ins(1,3,4,5)P_4 binding properties in NIH/3T3 and DT cells. Here, we report that [^{3}H]Ins(1,3,4,5)P_4 binding sites in both types of cells have properties similar to those of GAP1 (14,15). We also demonstrate the presence of transcripts which are detectable with mouse

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Abbreviations used: Ins(1,4,5)P_3, inositol-1,4,5-trisphosphate; Ins(1,3,4,5)P_4, inositol-1,3,4,5-tetrakisphosphate; DT cells, *ras*-transformed NIH/3T3 fibroblast cells; GAP, GTPase-activating protein; $InsP_4$, inositol tetrakisphosphate; Ins(1,3,4,5,6)P_5, inositol-1,3,4,5,6-pentakisphosphate; $InsP_6$, inositol hexakisphosphate; Ins(1,3,4,6)P_4, inositol-1,3,4,6-tetrakisphosphate; Ins(3,4,5,6)P_4, inositol-3,4,5,6-tetrakisphosphate.

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GAP1^m cDNA probes, but not with synaptotagmin cDNA, by Northern blot analysis of RNA from both types of cells.

MATERIALS AND METHODS

Materials. [^{3}H]Ins(1,3,4,5)P_4 (21 Ci/mmol) was obtained from New England Nuclear (Boston, MA). D-*myo*-Ins(1,3,4,5)P_4 and D-*myo*-Ins(1,4,5)P_3 were purchased from Dojindo Laboratories (Kumamoto, Japan). Other $InsP_4$, inositol-1,3,4,5,6-pentakisphosphate (Ins(1,3,4,5,6)P_5) and inositol hexakisphosphate ($InsP_6$) were from Sigma (St. Louis, MO).

Preparation of cell membrane fraction. NIH/3T3 mouse fibroblast and its ν-K-*ras*-transformed derivative (DT) cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (6). Cells were detached in Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline and collected by centrifugation. The cell pellet was mildly sonicated in a homogenizing buffer (50 mM Tris HCl, pH 7.0, 1 mM EDTA, 1 mM dithiothreitol, and 100 mg/l phenylmethylsulfonyl fluoride) and centrifuged for 10 min at 800 × g. The pellet was sonicated again in homogenizing buffer and centrifuged. The supernatants from these two steps were combined and centrifuged at 10,000 × g for 15 min and the resulting supernatant was again centrifuged at 100,000 × g for 60 min. Finally, the pellet was suspended in a binding assay buffer (100 mM KCl, 20 mM NaCl, 1 mM EDTA, 0.1% (w/v) bovine serum albumin, and 10 mM Hepes, pH 7.0). Protein concentration was determined according to the method of Bradford (16) with bovine serum albumin as the standard.

[^{3}H]Ins(1,3,4,5)P_4 binding studies. [^{3}H]Ins(1,3,4,5)P_4 saturation binding and displacement assays were performed in a total volume of 50 μl, each containing the above binding assay buffer and 0.1 mg of membrane protein. After incubation on ice for 20 min, bound and free [^{3}H]Ins(1,3,4,5)P_4 were separated by centrifugation for 15 min at 100,000 × g. The pellet was washed with the buffer solution and suspended in distilled water. The radioactivity was determined with a liquid scintillation counter. Amount of non-specifically bound ligand was determined by addition of 1 or 10 μM unlabeled Ins(1,3,4,5)P_4. Non-specific binding was minimized with the buffer at pH 7.0.

Northern blot analysis. Total cellular RNA was extracted from both types of fibroblasts and mouse brain as described previously (17), and poly(A)$^+$ RNA was separated by using Oligo(dT)-Latex (Takara Shuzo, Kyoto, Japan). RNA was electrophoretically separated on a 1.1% agarose-2.2 M formaldehyde gel and transferred onto a Zetaprobe nylon membrane (Bio-Rad, Hercules, CA). Hybridization was performed at 42 °C, as reported previously (8). The probes used were cDNA encoding amino acids 22-276 (probe GAP-A) and 164-276 (probe GAP-C) of mouse GAP1^m (15) labeled by the random primer method. cDNA encoding amino acids 139-393 of mouse synaptotagmin II (18) was also used. Filters were washed at 60 °C in 0.2 × SSC containing 0.2% SDS. Autoradiography was carried out at −80 °C with an intensifying screen for 14 days.

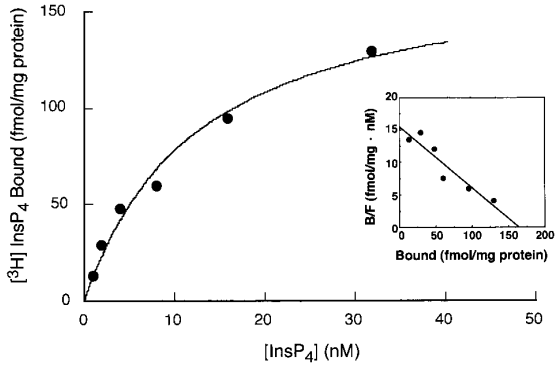

FIG. 1. Specific saturation binding of [^{3}H]Ins(1,3,4,5)P_4 to membrane fractions of *ras*-transformed NIH/3T3 (DT) cells. The membrane fraction prepared from DT cells was incubated with increasing concentrations of [^{3}H]Ins(1,3,4,5)P_4 in the presence and absence of 10 μM Ins(1,3,4,5)P_4. Inset shows Scatchard analysis of the data. Each point shown is the mean of three experiments.

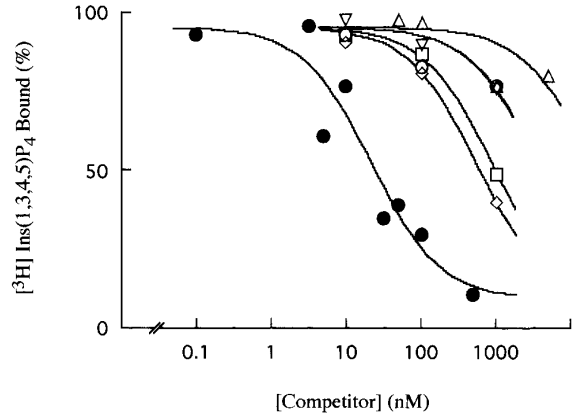

FIG. 2. Displacement by inositol polyphosphates of [^{3}H]-Ins(1,3,4,5)P_4 binding to DT cell membranes. The membrane fraction was incubated with 4.8 nM [^{3}H]Ins(1,3,4,5)P_4 in the presence of various concentrations of Ins(1,3,4,5)P_4 (●), Ins(1,3,4,5,6)P_5 (◇), Ins(3,4,5,6)P_4 (□), Ins(1,3,4,6)P_4 (○), $InsP_6$ (▽), and Ins(1,4,5)P_3 (△). Each point is the mean of two triplicate determinations. Data are expressed as the percentage of specific [^{3}H]Ins(1,3,4,5)P_4 binding in control.

RESULTS AND DISCUSSION

Ins(1,3,4,5)P_4 binding experiments were performed with membrane fractions of *ras*-transformed NIH/3T3 (DT) cells with increasing concentrations of [^{3}H]-Ins(1,3,4,5)P_4 in the presence or absence of 10 μM Ins(1,3,4,5)P_4. Ins(1,3,4,5)P_4 binding was saturable in DT cells as demonstrated in Fig. 1. A Scatchard analysis of [^{3}H]Ins(1,3,4,5)P_4 binding to DT cell membranes revealed a single class of binding sites, with a K*d* of 10.6 nM and a B*max* of 165 fmol/mg protein. [^{3}H]-Ins(1,3,4,5)P_4 binding sites in non-transformed NIH/3T3 cells showed similar affinity and density to those of DT cells: K*d*, 8.6 nM; B*max*, 121 fmol/mg protein (data not shown). These values in parental and *ras*-transformed cells were comparable to those reported in porcine platelets (12), smooth muscles (19), and rat hepatocytes (20).

Next, the ability of several inositol polyphosphates to displace [^{3}H]Ins(1,3,4,5)P_4 from binding sites in DT cell membranes was examined. The potency of competition was in the following order (Fig. 2): Ins(1,3,4,5)P_4 > Ins(1,3,4,5,6)P_5 > $InsP_6$ > Ins(1,4,5)P_3. This rank order is similar to that reported for GAP1^{IP4BP} (12, 21)

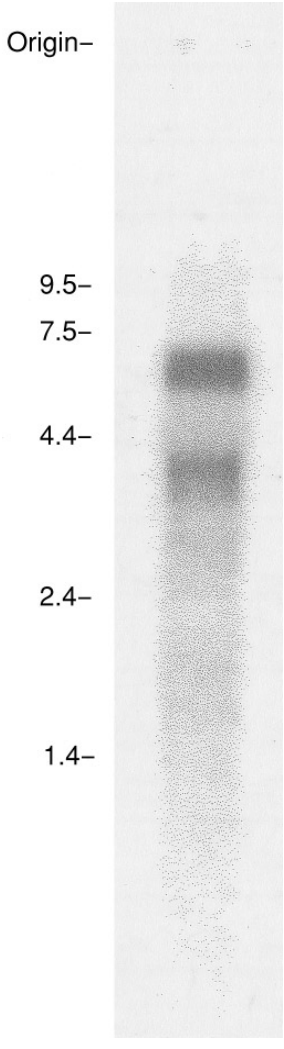

FIG. 3. Northern blot analysis of mRNA from DT cells. Poly $(A)^+$ RNA (4.4 μg) was separated by electrophoresis, blotted, and hybridized with a ^{32}P-labeled GAP1^m cDNA probe (GAP-C). Positions of size markers (in kilobases) are indicated on the left.

and for the $InsP_4$-binding domain of GAP1^m (15), but different from that of synaptotagmin II and AP-2 clathrin assembly protein (11,13). Affinity of two other naturally-occurring $InsP_4$ isomers, Ins(1,3,4,6)P_4 and Ins(3,4,5,6)P_4, to [^{3}H]Ins(1,3,4,5)P_4 binding sites of DT cell membranes was lower than that for Ins(1,3,4,5)P_4 but similar to that for Ins(1,3,4,5,6)P_5 (Fig. 2). This property is also the same as that of GAP1^{IP4BP} (12).

At present, three mammalian GAPs have been reported: GAP1^m (rat, 22; and mouse, 15), GAP1^{IP4BP} (human, 14) and GAPIII (rat, 23). We therefore examined whether or not control and *ras*-transformed fibroblasts express mRNA for mouse GAP1 as an Ins(1,3,4,5)P_4 binding protein by Northern blot analysis. cDNA probes derived from mouse GAP1^m (GAP-A and GAP-C) detected two RNA species of 5.6 and 3.6 kb in control (data not shown) and *ras*-transformed (Fig. 3) NIH/3T3 cells as well as in mouse brain (data not shown). A transcript of 7.0 kb hybridizable with synaptotagmin II cDNA was detected in mouse brain, but not in DT cells (data not shown).

The results show that parental and *ras*-transformed NIH/3T3 fibroblast cells possess high affinity Ins-(1,3,4,5)P_4 binding sites in their membrane fractions and express mRNA for GAP1^m. As reported in GAP1-^{IP4BP} (14), $InsP_4$ binding to GAP1-like $InsP_4$ receptors in these fibroblasts may increase Ras-GDP from Ras-GTP by hydrolysis. In contrast, tyrosine kinase(s) activated by bradykinin (8,9) may facilitate(s) the exchange to Ras·GTP from Ras·GDP. Such increased turnover of active or inactive states of Ras seems to be an important step for initiating Ca^{2+} influx. Viral Ras protein expressed in DT cells may function to lock the Ca^{2+} influx process at the open state, as suggested previously (6,8).

The results also show that Ins(1,3,4,6)P_4, Ins-(3,4,5,6)P_4 and Ins(1,3,4,5,6)P_5 interfere with [^{3}H]-Ins(1,3,4,5)P_4 binding similarly (Fig. 2). Our previous observation has demonstrated that Ins(1,3,4,6)P_4 and Ins(3,4,5,6)P_4 elicit Ca^{2+} influx, as Ins(1,3,4,5)P_4, while Ins(1,3,4,5,6)P_5 does not (3,6,7). Taken together, these results suggest that the Ins(1,3,4,5)P_4 receptor protein in fibroblasts could clearly discriminate $InsP_4$ from Ins(1,3,4,5,6)P_5. The exact reason why Ins(1,3,4,5,6)P_5 is invalid in Ca^{2+} influx is unknown at this moment.

In conclusion, the present results extend the notion that $InsP_4$ receptor protein (GAP1) may participate in regulation of Ca^{2+} influx not only in human platelets (24) but also in fibroblast cells.

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