Cloning and Tyrosine Phosphorylation of a Novel Invertebrate Immunocyte Protein Containing Immunoreceptor Tyrosine-based Activation Motifs

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Immunoreceptor tyrosine-based activation motif (ITAM) plays an important role in signal transduction through mammalian T-cell and B-cell antigen receptors and Fc receptors. The ITAM has been found only in vertebrate immunocytes. Ascidians are intriguing invertebrates and therefore are considered to be ancestors of the vertebrates. We have previously shown that the monoclonal antibody A74 inhibits cellular defense reactions of the ascidian. In the present studies, we found that the A74 antigen protein has two ITAMs and several motifs that are proposed to function in signal transduction. The A74 protein is tyrosine-phosphorylated and associated with other proteins in the initial stages of cellular defense reactions. The ITAMs of the A74 protein are tyrosine-phosphorylated by a c-Src kinase in vitro. 

In this paper we report the cloning of the A74 protein. We found that the A74 protein has two (one typical and one non-typical) ITAMs, which have been reported to play important roles in signal transduction through mammalian TCR, BCR, and FcRs. We also demonstrated that the ITAMs of the A74 protein are tyrosine-phosphorylated by a c-Src kinase. To our knowledge, this is the first finding concerning ITAM in invertebrates.

EXPERIMENTAL PROCEDURES

cDNA Cloning—The N-terminal amino acid sequence (3) of ascidian A74 protein was used to design degenerate oligonucleotide primers for PCR (5’-G(G/C)TTCA/GAT(T/C)AAC/G(CT/C)TG(A/G/C/G)AG/GC/CTA/GC/G(T/C)GC/CA/OG/GC/CTA/GC/G(T/C)GC/G(T/C)AT-3) and 5’-GG(A/G/C/T)AA/G(CT/C)GC/CA/OG/GC/CTA/GA/GA/TGA/GT/GC/CT-3). The primers at concentrations of 10 μM were mixed in PCR to amplify the H. roretzi hemocyte cDNA library. PCR was done in 10 μl Tris-HCl, pH 9.5, containing 1.5 mM MgCl2, 50 mM KCl, 0.1% Triton X-100, 4 mM deoxynucleotides, and 25 units/ml Taq DNA polymerase (Toyobo). After denaturation at 94 °C for 5 min, 30 cycles were run with annealing at 42 °C for 2 min, elongation at 72 °C for 3 min, and denaturation at 94 °C for 1 min. A DNA band migrating at about 50 base pairs was isolated, cloned into a pGEM-T vector (Promega), and transformed into JM109 cells. The A74 cDNA clones were picked from 1 × 106 clones of the Agt11 cDNA library by phage plaque hybridization (7) using the subcloned N-terminal 51-base pair DNA fragment as a probe. Seventeen independent positive clones were obtained and had inserts of the cDNA of 1.1–3.4 kilobases. The longest insert clone containing the N-terminal amino acid sequence and the 3’-end poly(A)’ tail was subcloned in the EcoRI site of the plBlue-script SK+ plasmid DNA. The clone was sequenced on both strands by deletion methods. The nucleotide sequence of the A74 insert cDNA fragment was determined by a Taq dye primer cycle sequencing kit (Applied Biosystems) using an ABI 373A DNA sequencing apparatus (Applied Biosystems).

Northern Blot Hybridization—The blotted membrane was hybridized with a 32P-labeled A74 cDNA probe. The lanes were washed under high stringency conditions (twice in 2 × SSC and 0.1% SDS at 65 °C for 15 min).

Immunoprecipitation—Aggregated hemocytes (1 × 106 cells) of H. roretzi (3) were washed with filtered artificial seawater and frozen in liquid N2. The frozen hemocytes were lysed by homogenization and stirring at 4 °C for 30 min in 3 ml of lysis buffer (50 mM Tris-HCl, pH 7.6, containing 0.1% Lubrol PX, 0.15 mM NaCl, 0.02% NaN3, 10% glycerol, protease inhibitors (10 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 0.2 mM diisopropylfluorophosphate, and 0.2 mM leupeptin), and phosphatase inhibitors (2 mM NaVO3 and 50 mM NaF)). The lysate was clarified by centrifugation at 12,000 × g for 10 min, and the resulting supernatant was subjected to immunoprecipitation by treatment with antibodies to A74.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank/EMBL Data Bank with accession number(s) AB007512.

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1 The abbreviations used are: ITAM, immunoreceptor tyrosine-based activation motif; TCR, T-cell antigen receptor; BCR, B-cell antigen receptor; FcR, Fc receptor; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; GST, glutathione S-transferase; SH2, Src homology 2; SH3, Src homology 3.
50 μl (wet volume) of A74 antibody-immobilized Sepharose (3) at 4 °C for 3 h and subsequently by centrifugation (50 × g, 1 min). The resulting immunoprecipitates were washed three times with the lysis buffer containing 0.5 M NaCl, treated with 0.1 ml of the sample buffer for SDS-PAGE for 5 min at 95 °C, and centrifuged. The resulting supernatant was boiled for 5 min after the addition of 5% 2-mercaptoethanol and was then subjected to SDS-PAGE and Western blotting with antiphosphotyrosine antibody PY54 (Afiniti) or PY20 (Leino Technologies). Mouse IgG-Sepharose was used as a control.

Expression of GST-ITAM Fusion Proteins—DNA fragments corresponding to the N-terminal nontypical ITAM (ITAM (N), amino acids 299–317), the C-terminal typical ITAM (ITAM (C), amino acids 356–375), and the two-repeated ITAMs (ITAM (N, C), amino acids 299–375) were amplified by PCR from the A74 cDNA using 32P-labeled A74 cDNA as a probe, a single isolate the A74 cDNA clone. On Northern blot analysis of the reduced glutathione in 50 mM Tris-HCl, pH 9.6, and were subjected to SDS-PAGE, followed by Western blotting with anti-phosphotyrosine antibody PY20 and also by protein staining with Coomassie Brilliant Blue R250.

RESULTS AND DISCUSSION

Cloning and Northern Blot Analysis—In a previous study (3), we purified the A74 protein from ascidian hemocytes and determined its N-terminal peptide sequence. The N-terminal sequence was used to design degenerate oligonucleotide primers for PCR of the H. roretzi hemocyte γgt11 cDNA library. The deduced amino acid sequences of the PCR products were found to include the N-terminal peptide sequence (3) of the A74 protein. The N-terminal 51-base pair PCR product was used as a probe for screening the hemocyte cDNA library and also with 20 mM Tris-HCl, pH 7.5, containing 2 mM MgCl2 and the solutions were incubated at 30 °C for 24 h with gentle shaking. Then 50 units/ml c-Src kinase (Upstate Biotechnology, Inc.) and 5 mM ATP were added to start the reaction, and the solutions were incubated at 30 °C for 3 h and subsequently by centrifugation (50 × g, 1 min). The result-
FIG. 2. Nucleotide and deduced amino acid sequences of A74 cDNA. The sequence is numbered from the first base at the 5' end. The polyadenylation signal, AATAAAA, is underlined. The deduced amino acid sequence matching the N-terminal sequence of purified A74 protein is

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lyzed tyrosine phosphorylation during hemocyte aggregation in *H. roretzi* (Fig. 4A). Transient tyrosine phosphorylation of two proteins of 260 and 160 kDa was observed 5–10 min after induction of hemocyte aggregation (4) followed by tyrosine phosphorylation of 90- and 75-kDa proteins, which indicates that there is different timing of tyrosine phosphorylation in the process of hemocyte aggregation. Tyrosine phosphorylation of the A74 protein was detected also during phagocytosis (data not shown). It should be noted that addition of the A74 antibody to the hemocyte suspension triggered tyrosine phosphorylation of the above-mentioned proteins including the A74 protein of 160 kDa, whereas the right panel shows the result on protein staining. B, the immunoprecipitates formed by treatment with the A74 antibody were subjected to SDS-PAGE followed by Western blotting with anti-phosphotyrosine antibody. The A74 protein of 160 kDa was tyrosine-phosphorylated and associated with 90- and 75-kDa proteins, both of which were also tyrosine-phosphorylated.

**FIG. 4.** Tyrosine phosphorylation during ascidian hemocyte aggregation. A, *H. roretzi* hemocytes collected in a process of hemocyte aggregation were subjected to SDS-PAGE, followed by Western blotting with the anti-phosphotyrosine antibody. The left panel shows time-dependent tyrosine phosphorylation of hemocyte proteins including the A74 protein of 160 kDa, whereas the right panel shows the result on protein staining. B, the immunoprecipitates formed by treatment with the A74 antibody were subjected to SDS-PAGE followed by Western blotting with anti-phosphotyrosine antibody. The A74 protein of 160 kDa was tyrosine-phosphorylated and associated with 90- and 75-kDa proteins, both of which were also tyrosine-phosphorylated.
Fig. 5. Tyrosine phosphorylation of ITAMs derived from the ascidian A74 protein. A, GST-ITAM fusion proteins. GST was ligated to the N-terminal nontypical ITAM (ITAM (N)), the C-terminal typical ITAM (ITAM (C)), or the twice-repeated ITAMs (ITAM (N, C)). B, tyrosine phosphorylation of GST-ITAM fusion proteins by human c-Src kinase. Three GST fusion proteins carrying ITAM (N), ITAM (C), and ITAM (N, C), which had been bound to glutathione-agarose beads, were incubated with (+) or without (−) human c-Src kinase (50 units/ml) in the presence of 5 mM ATP at 30 °C for 24 h. After elution from the beads with reduced glutathione, pH 9.6, the fusion proteins were subjected to SDS-PAGE, followed by protein staining (Coomassie Brilliant Blue; CBB) and also by Western blotting with the anti-phosphotyrosine antibody (PY20). GST was used as a control. Three GST-ITAM fusion proteins, but not GST alone, were tyrosine-phosphorylated by the c-Src kinase.

Tyrosine Phosphorylation of GST-ITAM Fusion Proteins—To obtain definitive evidence for the involvement in signal transduction of ITAMs derived from the A74 protein, we expressed three GST fusion proteins carrying the N-terminal nontypical ITAM, the C-terminal typical ITAM, and the twice-repeated ITAMs (Fig. 5A). We demonstrated that each of the two ITAMs present in the A74 protein was tyrosine-phosphorylated by human c-Src kinase in vitro (Fig. 5B). In addition, our preliminary result indicates that a tyrosine-phosphorylated protein of 75 kDa was bound to each of the tyrosine-phosphorylated ITAMs. Taken together, these results lead us to propose that the A74 protein is involved in the initial stage of signal transduction through tyrosine phosphorylation.

Evolutionary Implications—To understand the signal transduction cascade through the A74 protein, we compared the A74 protein-mediated ascidian immune systems with mammalian immune systems, in both of which the ITAM-containing proteins are involved. The mammalian immunocytes use the receptors of oligomeric structures composed of signal recognition and signal-transducing subunits (see Fig. 3A). The receptors catch the respective signals through the former subunits and transduce them through the latter subunits: The ITAM-containing TCR-induced signaling cascade triggers the activation of transcriptional regulators to induce gene expression of cytokines (16, 17), and the signal cascade through the ITAM-containing FcR induces phagocytosis (5), which suggests that the extracellular domain of A74 protein catches the respective signals in both reactions. In addition, our results provide evidence that the ITAM-containing intracellular domain of A74 protein plays a role in signal transduction. It seems reasonable to suppose that expression of putative immunity genes occurs in the A74 protein-mediated cellular responses in H. roretzi. Thus, in contrast with the mammalian oligomeric immunoreceptors, the A74 protein is a multifunctional single molecule involved in both signal recognition and signal transduction. This implication leads us to hypothesize that a single prototypic immunoreceptor might be separated into two parts, signal recognition and signal-transducing subunits in a process of the evolution. Further investigation of the functions of the respective domains of A74 protein will provide an important key to understanding the origin of vertebrate immune systems.

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