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Aggregation of the FcεRI in Mast Cells Induces the Synthesis of Fos-Interacting Protein and Increases Its DNA Binding Activity: The Dependence on Protein Kinase C-β*

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The ability of c-Fos to dimerize with various proteins creates transcription complexes which can exert their regulatory function on a variety of genes. One of the transcription factors that binds to c-Fos is the newly discovered Fos-interacting protein (FIP). In this report we present evidence for the regulation of the synthesis of FIP by a physiological stimulus. We found that the aggregation of the mast cell high affinity receptor for IgE (FcεRI) induced the synthesis of FIP and increased its DNA binding activity. Moreover, down-regulation of the isoenzyme protein kinase C-β (PKC-β) by a specific antisense phosphorothioate oligonucleotide resulted in profound inhibition of FIP-Fos DNA binding activity. Thus, aggregation of the FcεRI on mast cells elicits a PKC-β-dependent signaling pathway which regulates FIP-Fos DNA binding activity.

IgE-antigen (IgE-Ag)1-mediated activation of mast cells results in the phosphorylation of the cell surface IgE Fc receptor (FcεRI), priming the receptor for direct interactions with effector molecules that initiate diverse signal transduction pathways (1). These signaling pathways ultimately lead to the release of mast cell granule contents and the activation of genes encoding inflammatory products.

Some of these genes are known to be regulated by AP-1 activity (2, 3). The AP-1 is a complex of transcription factors which play an important role in mitosis and, in particular, the entry of cells into S phase (4–6). The AP-1 is comprised of a Jun homodimer or a Fos-Jun heterodimer. The Fos protein was found to be incapable of forming a homodimer but rapidly forms a highly stable complex via a leucine zipper interaction with the Jun protein or with selected members of the family of activating transcription factor/AMPH-responsive element-binding proteins (7). As a heterodimer, Fos and Jun activate transcription through the AP-1 binding site found in the promoter region of a variety of genes (2, 3). The binding of this heterodimer to its DNA binding site may in part be regulated by the phosphorylation of Jun. The phosphorylation of Jun requires both mitogen-activated protein kinase and protein kinase C (PKC) activities (8). Recently we found that the aggregation of the FcεRI, on the interleukin-3-dependent murine fetal liver derived mast cells (MC-9), increased the AP-1 DNA binding activity in these cells, a process found to be dependent on PKC activity. We also found that in IgE-antigen-activated MC-9 cells most of the newly synthesized c-Fos was bound to proteins other than Jun.

The USF family represents one class of proteins which can interact with Fos (9). The protein product of USF-2 is generally referred to as Fos-interacting protein (FIP) (10, 11). Members of this family recognize the DNA sequence of CACGTG (10). They are also characterized by the leucine zipper (Zip) and the helix-loop-helix (HLH) dimerization motifs that are found in various eukaryotic transcription factors and makes these proteins members of the basic region Zip (bZip) and/or basic region HLH (bHLH) families. Family members include TFE3, USF-1, USF-2, TFEB, AP-4, Myc, and Max (11). The presence of the bHLH/Zip functional domains permits the formation of homodimers and heterodimers among the family members. The formation of these complexes may be regulated by protein kinase activity (8).

Protein kinase C regulatory enzymes have been implicated in physiological processes such as membrane receptor function, cell differentiation, and proliferation (12). The functional diversity of PKC may in part be mediated by distinct isoenzymes that play an important role in both the activation and regulation of signaling pathways in the cell. Recent studies in rat basophilic leukemia cells, a rat analog of mucosal mast cells, begin to explore the role of these isoenzymes in mast cell function. Ozawa et al. (13) found that a full secretory response to antigen could be reconstituted by either PKC-β or -ε, but not by PKC-α or -δ. Our own experiments, in these cells, showed that the increased activity of PKC-β and -ε, activated by the aggregation of the FcεRI, resulted in the induction of c-Fos and c-Jun mRNA synthesis (14). Recently we also observed a PKC-dependent increase in the binding of the FIP-Fos complex to its target DNA in response to the aggregation of the FcεRI (9). In the present study we assess the relationship between the aggregation of the FcεRI, activation of PKC isoenzyme activity, synthesis of FIP protein, and the FIP-Fos DNA binding activity.

MATERIALS AND METHODS

Cell Culture—MC-9 cells (15), obtained from the American Type Culture Collection (Rockville, MD), were maintained in RPMI 1640 supplemented with 2 mM l-glutamine, 2 mM nonessential amino acids, 100 units/ml penicillin, 100 μg/ml streptomycin (Life Technologies, Inc.), 50 μM β-mercaptoethanol (Fisher), 10% fetal calf serum (Bio-Lab,
jersalem), and 20% WEHI-3 conditioned medium. Human KU812 cells, obtained from the American Type Culture Collection (Rockville, Maryland), were maintained in the same MC-9 cultured medium, however, without the WEHI-3 conditioned medium (16).

The treatments of Cells with Phosphorothioate Oligodeoxynucleotide—The MC-9 cells were incubated for 6 h in the presence or the absence of 20 ng/ml of PMA and 100 ng/ml of brefeldin A. This was followed by washing and transfered to flat-bottom 24-well plates (Nunc, Denmark) at a density of 1×10^6 cells/well in growth medium containing or not either 10 μM sense or antisense phosphorothioate oligodeoxynucleotide specific to the mouse calcium-dependent isoforms of PKC (c-PKC). The cells were then incubated at 37°C in 5% CO₂, and after 24 h a second addition of 10 μM of oligonucleotide was made. This was followed by 24 h incubation prior to triggering of the cells with IgE-Ag. In some experiments the cells were washed three times after the oligonucleotide treatment and incubated with only growth medium for 24 h at 37°C prior to supernatant collection. The oligonucleotides used were from the mouse c-PKC nucleotide sequence. The sense oligonucleotide was: 5′-GTACAGAT-GCAAAAAAT-3′ and the antisense oligonucleotide was: 5′-ATTTTTC-GCATCTCGTAC-3′ corresponding to bases 577–594, respectively (17).

Ig-E-Ag Stimulation of Mast Cells—MC-9 cells were transfected with serum-free RPMI medium. IgE sensitization was carried out by incubating duplicates of 2.5×10^5 cells/well with 2.5 μg of monoclonal IgE against dinitrophenol (DNP) (kindly provided by Dr. F.-T. Liu, La jolla, CA) for 45 min at 37°C in a final volume of 200 μl of modified Tyrode’s solution containing 10 μM Ca²⁺, 0.3 mM Mg²⁺ and 1 mg/ml gelatin (TG). The cells were then incubated at 37°C with 250 ng of DNP coupled to bovine serum albumin (DNP-BSA) for defined periods of time. Cells were quenched by washing in cold TG and recovered by centrifugation. Cell viability before and after each experiment was greater than 95% as determined by trypan blue exclusion.

Immunoblotting of PKC Isozymes—To assess the effect of the phosphorothioate oligonucleotides on PKC isozymes the treated cells were washed with saline and immediately solubilized by the addition of boiling Tricine-SDS sample buffer (Novex, Eincitais, CA) directly in the culture dishes. In a duplicate sample the cell number was determined. Proteins from the cell extracts were separated by electrophoresis on 10% SDS-PAGE and transferred to nitrocellulose membranes. Mouse monoclonal antibodies to the catalytic domain of PKC-α (Upstate Biotechnology, Inc., Lake Placid, NY) or of PKC-β (Seikagaku Corp, Tokyo, Japan) were used. An affinity-purified rabbit polyclonal antibody to the C terminus of rat PKC-β (Research & Diagnostics Antibodies, Berkeley, CA) was used at a 1:50,000 dilution. Rabbit polyclonal antibody to the C terminus of rat PKC-α (Santa Cruz Biotech, Santa Cruz, CA) was used at 1:100 dilution. The specificity of the antibodies used was confirmed using baculovirus expressed PKC-α, -β, -δ, -ε, -γ, and -η. Individual blots were incubated with the appropriate antibody overnight at 37°C in 4% milk phosphate-buffered saline. The immunoreactive proteins were detected using a species-specific, horseradish peroxidase-conjugated secondary antibody (Pierce, Rockford, IL). Autoradiography was by densitometric analysis.

MD-9 cDNA Library and DNA Sequencing—A cDNA library derived from IgE-stimulated MD-9 cells was prepared in the λ Zap vector (Stratagene). Screening of this library was by use of a polymerase chain reaction-generated probe derived from the human nucleotide sequence of FIP. The probe is a 284-base pair oligonucleotide fragment derived from the 3′ region of the human FIP cDNA (11). Primers used were sense, 5′-AGG AGA AGA GCC CAG CAC AAC-3′; corresponding to bases 676–693, and antisense, 5′-TGC TGG ACT GCC-3′, corresponding to bases 688–701. Five identical clones were isolated from 15×10^9 plaques screened. DNA sequencing was by the dideoxy termination method using double strand DNA templates and Sequenase (19) and G3-specific primers.

Preparation of Rabbit Anti-mouse FIP Antibody—A synthetic 19-mer peptide, QCHNLMEGVSETI, corresponding to the amino acid sequence encoded by the base pairs 180–216 of the open reading frame of the cloned mouse FIP was synthesized as a eight-branched peptide on multiple antigen peptide resin (ABI, Foster City, CA). A search of the SwissProt Data Bank for FIP peptide homology did not reveal a protein with greater than 20% homology to this peptide. The primary immunization of rabbits was intramuscular with subsequent subcutaneous immunizations of 1 mg of peptide mixed with Ribi adjuvant (RIBI ImmunoChem Research, Inc.). Rabbits were boosted two times in 14-day intervals with the same amount of peptide. Seven days after the last boost animals were bled, and the presence of antibodies to FIP was determined.

Serum antibodies to the synthetic peptide were assayed by enzyme-linked immunosorbent assay using 20 μg of peptide and 2 μg of BSA as control per well. Both preimmune and immune sera were diluted by serial 10-fold dilutions to a final of 1:10. The endpoint titer was defined as the index of absorbance that gave values of absorbance of at least 0.2, these values were 10 times higher than the mean background absorbance for BSA. Detection of bound antibody was by using anti-rabbit IgG coupled with alkaline phosphatase and 1 mg/ml p-nitrophenyl phosphate as substrate for colorimetric detection at 405 nm. Depletion of antibody reactivity was achieved by preincubating the serum absorb at dilutions of 1:4 with excess peptide prior to the assay. This resulted in an 80% reduction in antibody reactivity to peptide antigen.

Gel Shift Assays—Cell extracts were prepared from 10⁵ cells and used in gel shift assays as described previously (20). Synthetic double-stranded TRE and FIP binding site (FBS) oligonucleotides were used for this assay. The following sequences were used: TRE, 5′-AGC TTA AAA AAG CAT GAG TCA GAC ACC TG-3′ and FBS, 5′-GAT CCT AGG CCA CGT GAC CGG-3′. The bound proteins were identified by using a rabbit polyclonal antibody to c-Fos (Medac, Hamburg, Germany) and the rabbit polyclonal anti-mouse FIP described above.

Biosynthetic Labeling and Immunoprecipitation—After IgE sensitization, cells were washed once with Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum and 1 mg/ml gelatin (TG). The cells were then incubated at 37°C with 250 ng of DNP coupled to bovine serum albumin (DNP-BSA) for defined periods of time. Cells were quenched by washing in cold TG and recovered by centrifugation. Cell viability before and after each experiment was greater than 95% as determined by trypan blue exclusion.

Immunoprecipitation was essentially as described previously (21) with the following modification: 2.5×10⁵ cells were lysed by the addition of 500 μl of cold lysis buffer (0.01 M Tris-HCl, pH 7.4, 1% deoxycholate, 1% Triton X-100, 0.1% SDS, 0.15 M NaCl, and 0.25 mg/ml phenylmethylsulfonyl fluoride). Cells were subsequently homogenized, and supernatants were collected after 30 min of microcentrifugation at 4°C. Antibody to mouse FIP, which had been preincubated in the absence or presence of the FIP peptide, was added to the supernatants. After an overnight incubation at 4°C, 10 mg of protein A-Sepharose was added, and the mixture was incubated for 3 h at 4°C with mixing. The recovered immunoprecipitate was washed three times with lysis buffer and once with Tris-EDTA. Proteins were solubilized in boiling Laemmli sample buffer containing 0.5% SDS. The proteins were resolved by 10% SDS-PAGE. Gels were dried and exposed to Kodak X-Omat AR film at −70°C for varying times. Quantitation of the autoradiography was by densitometric analysis.

RESULTS

Presence of FIP in MC-9 Cells—We were able to confirm the presence of FIP protein in MC-9 cells by incubating 35S-labeled cell lysate with the antibody to FIP which had been preincubated in the presence or in the absence of the immunogenic peptide (Fig. 1A). Immunoprecipitation revealed a predominant band with the apparent molecular mass of 49 kDa. No bands were detected when the sera used for immunoprecipitation was derived from the preimmune rabbit (data not shown). Specificity of the antibody was further determined by immunoprecipitation from 35S-labeled cell lysate of the human basophilic precursor cell line KU-812 (16) and the MC-9 cells. Fig. 1B shows that this antibody could recognize only the mouse FIP and not human FIP. In these initial experiments using equal numbers of cells, we observed that more FIP protein was immunoprecipitated from IgE-Ag-activated cells than from nonactivated cells (Fig. 1A), suggesting the possible regulation in response to activation.

Regulation of FIP Synthesis in Response to FcRI Engagement—Kinetic analysis of the synthesis of FIP protein in response to aggregation of the FcRI is shown in Fig. 2. The addition of DNP-BSA to IgE-sensitized cells results in a detectable increase in the levels of FIP within 15 min of addition with maximal levels reached 30 min after antigen addition (4-fold above control). These levels were found to be sustained for at least 120 min after addition of antigen (Fig. 2). This time course of FIP synthesis correlated with our previous results on the
increased binding activity of Fos-containing complexes to the FIP DNA binding site (9).

Identification of FIP in the FBS-binding Fos Complex—To confirm that FIP is included in the protein complex which binds to the FBS oligonucleotide, a gel shift assay was performed. As shown in Fig. 3 a supershift was obtained when extracts of MC-9 cells were preincubated with the rabbit polyclonal antibody to mouse FIP which had been preincubated in the absence or presence of FIP immunogen peptide prior to the addition of the antibody to the cell lysates. Immunoprecipitates were analyzed by SDS-PAGE. A supershift was also obtained when extracts of MC-9 cells were preincubated with rabbit antibody to mouse FIP and analyzed by SDS-PAGE. Each sample was run in duplicate. One representative experiment out of three is shown.

PKC Regulation of FIP-Fos DNA Binding Activity—We previously described that FIP-Fos and AP-1 DNA binding activities are regulated by PKC (9). In the present study we investigated which PKC isoenzyme might be involved in the regulation of FIP DNA binding activity. We first determined the concentrations of PKC-α, -β, -δ, -ε, and -ζ in IgE-Ag activated or nonactivated MC-9 cells. We focused on these isoenzymes since they were previously reported to be present in cells derived from hematopoietic tissue (22) and in mast cells in particular (13). The intracellular concentration of each isoenzyme in resting cells is shown in Table I. No difference in isoenzyme concentration could be found after IgE-Ag activation of the cells. Protein kinase C-ζ was barely detectable in our immunoblots and could not be quantitated. Concentrations were determined by comparison to known concentrations of baculovirus expressed PKC isoenzymes in Western blots using quantitative densitometry under linear conditions. The predominant PKC isoenzymes present in the MC-9 cells were
PKC-\(\beta\) and PKC-\(\alpha\). The concentration of PKC-\(\beta\) was greater than 2-fold of that of PKC-\(\alpha\). The concentration of both PKC-\(\delta\) and -\(\epsilon\) were approximately 60-fold less than that of PKC-\(\beta\). ImmunobLOTS also revealed a down-regulation of all PKC isoenzymes (>90%) by PMA after treatment of the cells for 6 h (data not shown). This was found to be reversible after washing the cells and incubating for 24 h in medium free of PMA.

To study which of the PKC isoenzymes is involved in FIP-Fos and AP-1 DNA binding activities, the PKC isoenzymes were down-regulated by 6 h of treatment with PMA, followed by incubation with an antisense oligonucleotide, directed to the C2 domain of c-PKC (12), for an additional 48 h. ImmunobLOTS revealed a decrease of 65% - 16% (n = 3) in the levels of PKC-\(\beta\) (Fig. 4). Only a slight decrease in the levels of PKC-\(\alpha\) was observed, while PKC-\(\delta\) and -\(\epsilon\) levels were unaffected. Controls treated with sense oligonucleotides were minimally affected (Fig. 4). To determine the role of PKC-\(\beta\) in the DNA binding activities of FIP-Fos and AP-1, gel shift assays were performed using either a synthetic double-stranded TRE or FBS. We initially confirmed our previous results that aggregation of the FosRI on MC-9 cells could cause an increase in the DNA binding activities of both FIP-Fos and AP-1 complexes (9). Kinetic analysis of the DNA binding activity revealed that both FIP-Fos (Fig. 5) and AP-1 (data not shown) binding reached a maximum at 15-30 min after antigen stimulation. When PKC-\(\beta\) was down-regulated, FIP-Fos DNA binding activity decreased by an average of 50% (n = 6) in IgE-Ag-stimulated MC-9 cells (Fig. 6A). In contrast, no effect was observed on the binding activity of AP-1 in the PKC-\(\beta\)-depleted cells (Fig. 6B).

These results suggest that the aggregation of the FosRI in mast cells induces a PKC-\(\beta\)-dependent signaling pathway for FIP-Fos DNA binding activity.

Regulation of FIP Synthesis by PKC-\(\beta\)—To further explore the mechanism involved in the PKC-\(\beta\)-dependent decrease of FIP-Fos DNA binding activity, we measured the expression of FIP in IgE-Ag-activated cells pretreated with PKC-\(\beta\) sense or antisense oligonucleotides. A significant decrease in the expression of FIP was found in IgE-Ag-activated cells which were treated with the antisense oligonucleotide (Fig. 7A). No decrease was observed in sense oligonucleotide treated cells. Therefore, the decrease in the FIP-Fos DNA binding activity in...
activated cells where PKC-β was down-regulated could potentially be attributed to the reduction in the concentration of FIP. Since total recovery of PKC-β expression could be achieved after washing of PMA and removal of antisense oligonucleotides (data not shown), we assessed the effect of PKC-β recovery on the expression of FIP. Twenty-four hours after removal of the PKC-β antisense oligonucleotide from the culture medium, the concentrations of FIP returned to the normal levels observed in control and sense oligonucleotide-treated cells (Fig. 7B). These results suggest that PKC-β either directly or indirectly regulates the expression of FIP in the MC-9 mast cells.

**DISCUSSION**

Our previous studies have described the possible involvement of FIP in regulating AP-1 activity in MC-9 cells when these cells are activated by IgE-Ag (9). The analysis of the role of FIP was limited in these studies by the unavailability of antibody to FIP. Nevertheless, we defined the presence of FIP complexed to Fos by analyzing the ability of a Fos-containing complex to bind to the FIP-DNA binding site (9), since Fos alone does not bind to this DNA sequence. In the present study we show that FIP is present in the MC-9 cells by first identifying a FIP clone with 100% homology to mouse USF-2 (10) from a cDNA library derived from IgE-Ag-stimulated MC-9 cells. Second, we identify the presence of FIP protein with antibody generated to a FIP-specific peptide sequence (Fig. 1).

We further demonstrate that IgE-Ag stimulation of MC-9 cells regulates the synthesis of FIP protein and that PKC-β regulates both the synthesis of FIP and its DNA binding activity.

Although PKC-β was found to be the predominant isoenzyme in MC-9 cells, differential localization of the various PKC isoenzymes may lead to their involvement in the regulation of FIP expression and DNA binding activity. Moreover, the relative enzymatic activity of the individual PKC isoenzymes does not necessarily correlate with their intracellular concentrations. Thus, we cannot exclude the possibility that other isoenzymes of PKC may also participate in regulating the expression and DNA binding activity of FIP. However, our results are conclusive for a role of PKC-β activity in either directly or indirectly regulating the synthesis of FIP and of FIP DNA binding activity in response to the aggregation of the FcεRI.

Protein kinase C-β plays a major role in the secretory response of mast cells (13) and also links the FcεRI to the induction of AP-1 component expression (14) in the rat basophilic leukemia cell line. However in rat basophilic leukemia cells, PKC-α is the predominant isoenzyme (14), while in the murine MC-9 cells, PKC-β is the most highly expressed. These differences may be species-specific (rat versus mouse), influenced by the state of differentiation, state of retroviral/chemical transformation, or growth conditions, yet PKC-β regulates transcription factor synthesis in both cell lines. Nevertheless it still remains unclear as to how PKC-β is capable of regulating the secretory response, expression of FIP, and expression of AP-1 components in mast cells. It could be argued that the calcium influx in response to the aggregation of the FcεRI leads to the preferential activation of the calcium-dependent PKC-β. In contrast, activation of the calcium-independent PKC-ε, which also is capable of stimulating c-Fos and c-Jun expression but inhibits phosphatidylinositol hydrolysis (23), might be attenuated in vivo by negative regulatory influences in response to aggregation of the FcεRI. Furthermore, differences in the amino acid sequence of these isoenzymes may determine their interactions with other proteins in vivo which may play a role in the regulation of their activity and thereby of cellular responses.

Based on the results of this study we conclude that the induction of FIP synthesis and increased FIP-Fos DNA binding activity, by aggregation of the FcεRI, requires the activity of PKC-β. According to our results the decrease in FIP-Fos DNA binding activity in the antisense oligonucleotide-treated cells could at minimum be partially attributed to the reduction in the level of FIP in these cells. The ability of Fos to bind to other nuclear proteins such as FIP may regulate transcription in a receptor-specific manner. Furthermore, formation of receptor-specific complexes may serve to regulate gene transcription in a direct and indirect manner. For the latter, it is well known that the affinity of binding of Fos-Jun to the TRE is greater than that of Jun-Jun (2). Thus, the formation of FIP-Fos complexes may attenuate the activity of the AP-1 by decreasing the amount of Fos that might be available for association with Jun. This, the receptor-specific synthesis of FIP would indirectly control genes whose expression was regulated by AP-1. Protein kinase C-β would also influence this transcriptional regulation by increasing the formation of FIP-Fos complexes and increasing the DNA binding activity of this complex.

The specificity of the antisense oligonucleotide approach was determined in our system by the specific inhibition of the expression of only PKC-β and not other isoenzymes and by the inability of sense oligonucleotides (24) to cause a similar effect. However, we cannot exclude the possibility that the effect observed in this study was due to an indirect effect of the
antisense oligonucleotides that ultimately resulted in the down-regulation of PKC-β. As previously mentioned the antisense oligonucleotide was designed to the C2 domain responsible for the calcium dependence of c-PKC (12). The amino acid sequence of this site is 90% homologous between the murine PKC-α and -β (17, 25). Despite the high homology there was only a minimal decrease in the level of PKC-α, while the expression of PKC-β was decreased by greater than 60%. This preferential effect of the antisense might be explained by the difference in the concentrations of PKC-α and -β in MC-9 cells. Thus, since PKC-β is present in these cells at a concentration greater than 2-fold that of PKC-α, it is possible that the oligonucleotide which entered the cells could be preferentially bound by the PKC-β mRNA. Alternatively, the preferential binding of the oligonucleotide to PKC-β might be explained by differences in the variable regions adjacent to the C2 domains of these isoenzymes that may influence the conformation and availability of these domains.

Examples for the cross-talk between signaling pathways in biological systems are growing in number. The ability of c-Fos to bind to various proteins enables the cross-talk between the enzymes that are activated by receptor stimulation and genes that are activated or inactivated as a consequence of a particular stimulus. It has to be determined whether other stimuli, like growth factors, might initiate an association of c-Fos with c-Jun rather than with FIP. Furthermore, it is possible that the PKC isoenzymes involved in increasing the DNA binding activity of the AP-1 complex differ from that which initiate binding at the FBS. This complexity in regulation of gene expression might be expected in light of the diverse responses initiated by diverse stimuli.

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