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Intracellular Signaling Pathways for Type II IgE Receptor (CD23) Induction by Interleukin-4 and Anti-CD40 Antibody

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Abstract : Since the role of CD40 on the interleukin-4(IL-4)-induced B cell activation has been strongly implicated in the agumentation of IgE production and response, we have investigated the intracelluar signaling pathways utilized by IL-4 and CD40 for type II IgE receptor (CD23) expression. IL-4 and anti-CD40 antibody treatment of human B cells, independently caused a rapid induction of CD23 gene activation within 2 h. There was a noticeable synergism between the action of the two agents inducing CD23 expression; the addition of anti-CD40 to the IL-4-treated culture significantly agumented the IL-4-induced CD23 on both mRNA and surface protein levels, and the inclusion of IL-4 in the anti-CD40-treated cells caused a further increase of CD23 expression far above the maximal level induced by anti-CD40. Protein tyrosine kinase (PTK) inhibitors effectively suppressed the both IL-4- and anti-CD40-induced CD23 expression, whereas protein kinase C (PKC) inhibitors had no effects. Electrophoretic mobility shift assays (EMSA) have shown that IL-4 and anti-CD40 induce the activation of NF-IL-4 and NF-$_{\kappa}$B, respectively, binding to the CD23 promoter, both in a PKC-independent and PTK-dependent manner. These data suggest that the synergistic activation of CD23 gene expression by IL-4 and anti-CD40 is mediated by co-operative action of distinct nuclear factors, each of which is rapidly activated via PKC-independent and PTK-dependent process.
Key words : CD23, CD40, interleukin-4, signal transduction

While helper T (Th) cell-derived cytokines play an important role in the regulation of immunoglobulin isotype switching during B cell activation and differentiation, it is now well established that the interaction between B cell surface molecule CD40 and its ligand (CD40 L) on activated Th cells provides a co-stimulatory signal necessary for the T cell-dependent isotype switching (Armitage *et al.* 1992; Noelle *et al.*, 1992; Foy *et al.*, 1993) Whereas *in vitro* production of IgE in the purified B cell culture by IL-4 alone has been difficult to obtain, human and mouse B cells can be indeed induced to undergo isotype switching *in vitro*, primarily to IgG1 and IgE by the treatment with the combination of IL-4 and anti-CD40 or recombinant CD40 L(Gascan *et al.*, 1991; Russet *et al.*, 1991; Splawski *et al.*, 1993; Warren and Berton., 1995). CD23, expressed on activated B cells is thought to serve as a cell surface marker for terminally differentiated B cells which are programmed to secrete IgE (Delespesse *et al.*, 1989; Conrad, 1990). As the low affinity receptor for IgE (Fc ε RII), the role of CD23 has been strongly suggested in the IgE-mediated antigen uptake, processing and presentation, thereby inducing Th2 cell activation and enhanced production of IL-4, implicating CD23 as a central mediator of the IL-4 autocrine loop involving IL-4 → IgE/CD23 → IL-4 (Kehry and Yamashita 1989; Mudde *et al.*, 1995). Despite such critical roles played by CD23 in the IL-4-mediated immune response, molecular mechanisms of CD23 regulation by IL-4 during B cell activation have not been elucidated. In a previous study, we have shown that in quiescent human B cells CD23 is a primary response gene rapidly induced by IL-4 within 1-2 h, in the absence of on-going protein synthesis (Lee *et al.*, 1993), which strongly suggests that CD23 gene induction system can serve as a good target for probing the IL-4 signal transduction. In the present study we have investigated the intracelluar signaling pathways utilized by IL-4 and anti-CD40 for CD23 expression in human primary B cells. Our data clearly demonstrate that the signals mediated by IL-4 receptor and CD40 are delivered via independent pathways to cause synergistic induction of CD23 expression both at mRNA and surface protein levels. The rapid response of CD23 gene activation upon IL-4 and anti-CD40 stimulation appears to involve activation of distinct nuclear factors, NF-IL-4 and NF-$_{\kappa}$B, respectively, binding to distinct

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sites on the CD23 promoter, each of which is regulated by the action of protein tyrosine kinase but not of protein kinase C (PKC). The ability of IL-4 and anti-CD40 to synergize to induce CD23 expression as an immediate early (primary response) gene via tyrosine kinase-mediated signaling pathways would provide a signal co-operation system to stimulate B cell activation process, which in turn leads to the final stage of B cell differentiation, production of IgE via isotype switching.

Materials and Methods

Human B lymphocyte isolation and culture

Human B cells were prepared from freshly excised tonsils by Ficoll-Hypaque (Sigma, d=1.077) density gradient centrifugation and negative selection upon rosetting twice with AET-treated sheep RBC. After removing adherent cells, about 95% purity of B cells was obtained as confirmed by staining with anti-Leu16, anti-Leu4 or anti-Leu M5 (Becton Dickinson). Purified B cells were cultured in RPMI medium (Sigma) containing 10% FBS (Hyclone), 10 mM Hepes, 2 mM L-glutamine, 5×10^{-5} M 2-mercaptoethanol, and antibiotics. Recombinant human IL-4 expressed in *E.coli* (Yang *et al.* 1992), PMA, or monoclonal anti-CD40 antibody 5C3 (Dr. Kikutani, Osaka University) were added to cells as indicated. Protein kinase inhibitors were treated to cells for 30 to 60 min prior to the addition of IL-4 or anti-CD40.

Analysis of CD23 mRNA expression

Total cellular RNAs from B cells (1×10^8 cells) were isolated after treatment with IL-4, anti-CD40 or PMA for various durations as indicated, using guanidinium isothiocyanate and cesium chloride through ultracentrifugation (Chirgwin *et al.*, 1979). For Northern blot, 10 μg of total RNAs from each preparation were separated on a 1% agarose-formaldehyde gel, transferred to nylon membranes (Genescreen Plus, NEN), and hybridized with [^{32}P]-labeled full-length CD23 cDNA probe (from Dr. Kishimoto, Osaka University) at specific activity of 5×10^8 cpm/μg, prepared using a nick translation kit (Amersham). RNA concentration was determined by OD measurement and the amount of loaded RNA on the gel was confirmed by ethidium bromide (EtBr) staining.

Analysis of membrane CD23 expression by flow cytometry

CD23 expression on cultured B cells (1×10^6 cells) was analyzed by staining with a mouse monoclonal anti-human CD23-FITC (Immunotech) in Hank's balanced salt solution containing 3% FBS 0.1% NaN_3 for 30 min at 4°C, using fluorescence-activated cell scanner (FACScan, Becton-Dickinson). Surface CD23 levels were expressed as the mean fluorescence intensity (MFI) for CD23. △MFI was calculated as MFI of anti-CD23-FITC-stained cells-MFI of control mouse IgG-stained cells. The values represent a mean of three independent determinations and SD≤10% of the mean, unless otherwise indicated.

Electrophoretic mobility shift assay (EMSA)

The purified tonsillar B cells (1×10^8) were treated with IL-4, anti-CD40 or IL-4 plus anti-CD40 in the presence or absence of various inhibitors of protein kinases for 20 min. Cells were harvested and processed for nuclear extract preparation as described by Dignam *et al.* (1983). The IL-4RE (Köhler and Rieber, 1993) oligomers and NF-$_\kappa$B oligomers containing -230~-214 bp and -265~-251 bp region of human CD23(b) promoter sequence (Yokota *et al.*, 1988), respectively, were custom-synthesized and purified by PAGE (Bioneer). The oligomers were end-labelled using [α-^{32}P]dCTP (NEN s.p.>3000 μCi/mmol) and Klenow. The binding of nuclear extract (5 to 10 μg) was performed in a reaction mixture containing 10 mM Tris-Cl (pH 7.5), 50 mM NaCl, 10 mM, $MgCl_2$, 1 mM DTT, 1 mM EDTA, 10% glycerol, 1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM, sodium vanadate, 1 mM sodium pyrophosphate, 10 mM sodium fluoride, 2 μg poly(dI)-(dC), 2 μg salmon sperm DNA, and 0.2 ng of labelled oligomers for 20 min at room temperature. The ananlysis of oligomer mobility was carried out by electrophoresis on a 6% acrylamide/0.5% TBE gel, followed by autoradiography.

Results and Discussion

IL-4 and anti-CD40 rapidly induce CD23 gene expression

The reported costimulatory action of anti-CD40 on the IL-4-induced B cell activation and differentiation has suggested that there may be a cross-talk between signals mediated by IL-4 receptor and CD40 of the B cell membrane (Valle *et al.*, 1989; Lee *et al.*, 1995). Since the induction of CD23 is a major phenotypical change caused by IL-4 in B cells (Defrance *et al.*, 1987), and our previous study has revealed that CD23 is a primary response gene whose mRNA expression is regulated via early signals delivered by IL-4 (Lee *et al.*, 1993), we examined the effect of anti-CD40 stimulation on the CD23 gene expression as a means to assess the B cell activation potential of anti-CD40 in conjunction with the IL-4 action. As shown in Fig. 1, treatment of tonsillar B cells with IL-4 (lane 2) or mitogen PMA (lane 4) for 2 h, caused a significant induction of CD23

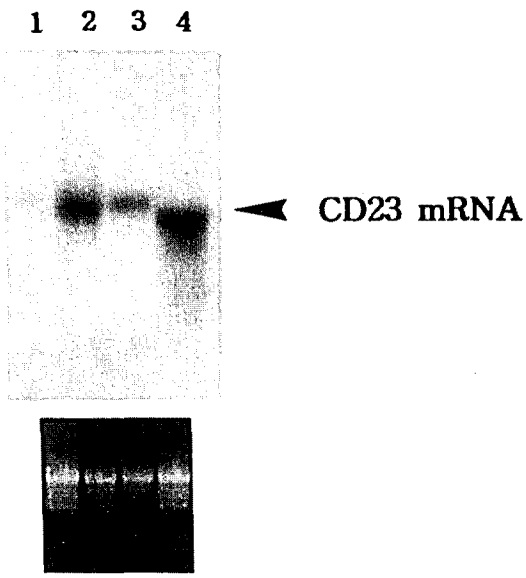

Fig. 1. Induction of CD23 mRNA by IL-4 and anti-CD40. Purified tonsillar B cells were stimulated with media (lane 1), 200 u/ml IL-4 (lane 2), 2 μg/ml anti-CD40 (lane 3) or 40 ng/ml PMA (lane 4) for 2 h, then cells were harvested for RNA isolation and Northern blot analysis was performed as in Materials an Methods. Top: Northern blot autoradiogram, Bottom: EtBr-stained RNA gel.

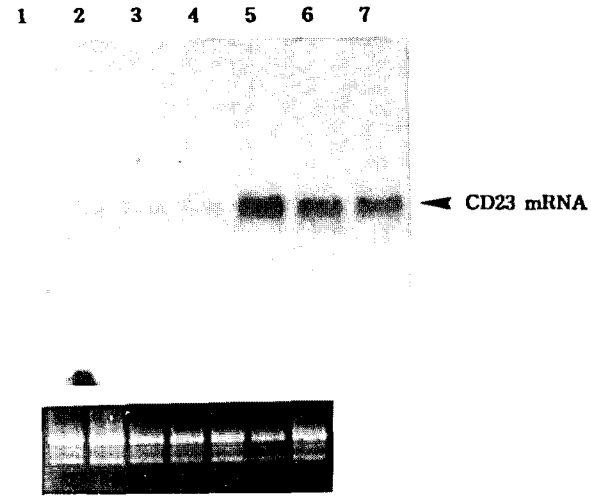

Fig. 2. Synergistic effect of IL-4 and anti-CD40 on CD23 mRNA induction. Purified B cells were treated with IL-4 and anti-CD40 and cultured for 12 h. Total cellular RNAs were isolated and subjected to Northern blot. Lane 1, control; lane 2, IL-4 50 U/ml; lane 3, IL-4 100 U/ml; lane 4, IL-4 200 U/ml; lane 5, IL-4 100 U/ml+anti-CD40 0.5 μg/ml; lane 6, IL-4 100 U/ml+anti-CD40 1.0 μg/ml; lane 7, IL-4 100 U/ml+anti-CD40 2.0 μg/ml. Top: Northern blot autoradiogram. Bottom: EtBr-stained RNA gel.

mRNA, confirming our earlier data. Stimulation of these cells with agonistic anti-CD40 antibody (5C3) at a concentration (2 μg/ml) known to induce B cell proliferation also caused a noticeable accumulation of CD23 mRNA within 2 h (lane 3), albeit lower than the level induced by IL-4. Such early response of gene expression suggested that, as in the case of the IL-4-induced CD23 mRNA, the induction of CD23 mRNA by anti-CD40 occurs through the activation of pre-existing factors and does not require a newely synthesized protein. This notion has been confirmed by the observation that pretreatment of cells with cycloheximide, a translational inhibitor does not adversely affect the anti-CD 40-induced CD23 gene expression (data not shown). The result indicates that signals delivered via CD40 produce effects similar to those of IL-4 on the rapid induction of CD23 gene expression and that CD23 is one of early response genes induced by anti-CD40 stimulation in human B cells.

IL-4 and anti-CD40 act synergistically to induce CD23 expression both at mRNA and surface protein levels

Having observed that anti-CD40 independently induces CD23 gene expression in a manner similar to that of IL-4, we then examined whether two agents can act co-operatively to agument the each other's response. It has been reported that IL-4 induces CD23 mRNA accumulation in a dose-dependent manner in human B cells (Lee *et al.*, 1993), As shown in Fig. 2, the IL-4-induced CD23 mRNA level tends to reach the saturation level at 100 u/ml of IL-4. However, the addition of anti-CD40 to the IL-4-treated culture caused a further increase in CD23 mRNA level. A similar agumentation was observed when IL-4 was added to the cells which express CD23 mRNA at a maximal level inducible by anti-CD40 treatment alone (data not shown). Such agumentation of CD23 expression by IL-4 and anti-CD40 was more prominent at the surface protein level as shown in Fig. 3. IL-4 and anti-CD40 each induces the surface CD23 expression on B cells in a dose-dependent fashion (Panel A-a and A-b), athough the overall induction level of surface CD23 expression by anti-CD40 is much lower than that by IL-4, which correlates with the mRNA data in Fig.1. The induced level becomes saturated at the IL-4 concentration of 100~200 u/ml (Panel B) or at the anti-CD40 concentration of 1~2 μg/ml (Panel C). Addition of anti-CD40 to the IL-4-treated culture or addition of IL-4 to the anti-CD 40-treated culture, however, profoundly enhances the CD23 expression induced by either agent alone (Panels A, B, and C). These data strongly indicate that IL-4 and anti-CD40 each utilizes distinct intracelluar signaling pathways to regulate CD23 expression, such that even after one pathway is saturated by the maximal stimuli exerted by one agent, the other agent can utilize another pathway to further stimulate CD23 gene ex-

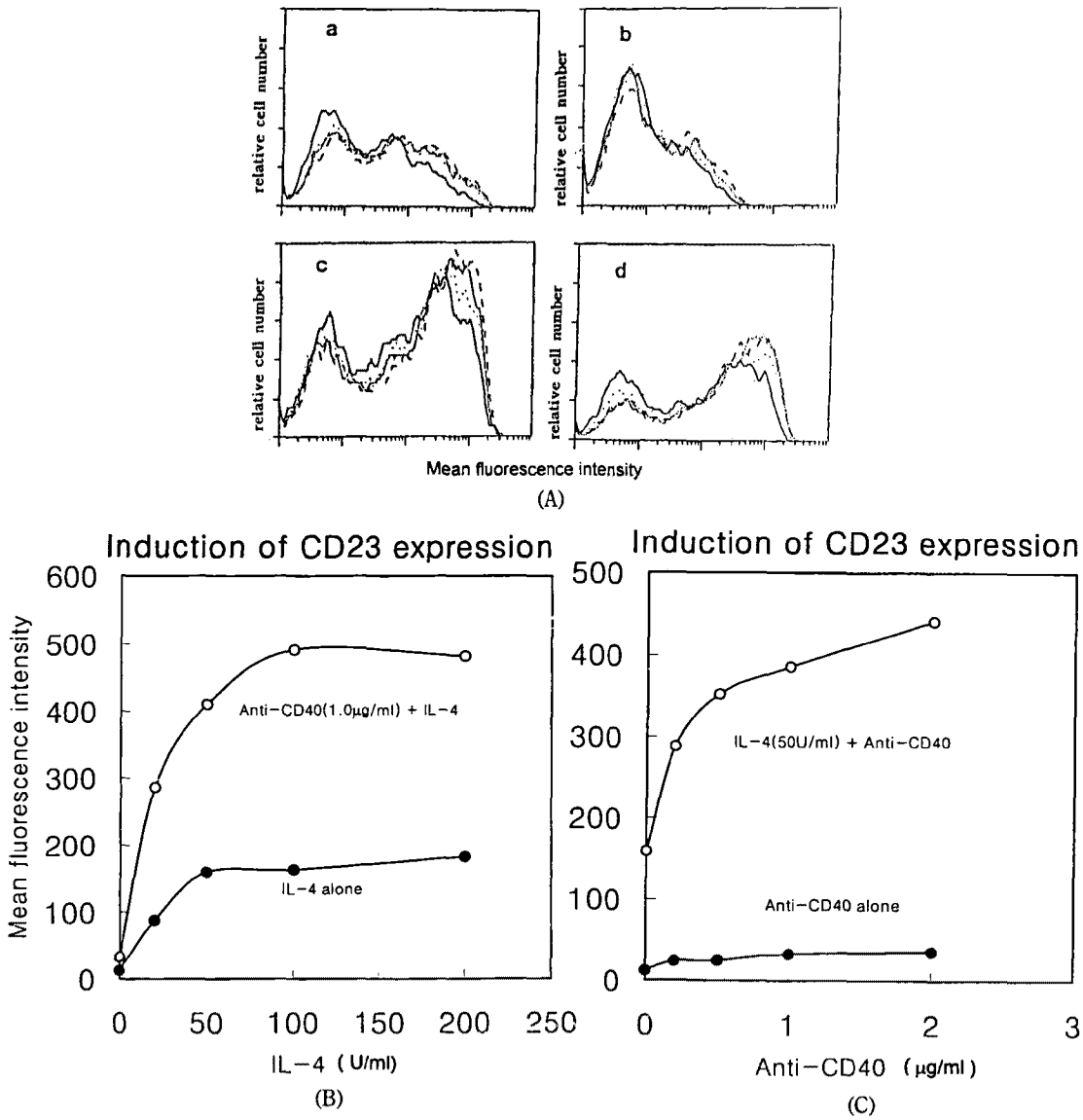

Fig. 3. Synergistic effect of IL-4 and anti-CD40 on the surface CD23 expression. Purified B cells were cultured in the presence of IL-4 and anti-CD40 for 24 h. Cells were then stained with anti-human CD23-FITC and analyzed by flow cytometry as in Materials and Methods. Panel A: A representative of FACScan histograms from several expriments is shown. △MFI for untreated control cells was 12. a: IL-4 20 U/ml (———, △MFI=87), IL-4 50 U/ml (· · · · , △MFI=159), IL-4 100 U/ml (·····, △MFI=162), IL-4 200 U/ml (- - -, △MFI=182), b: anti-CD40 0.2 μg/ml (———, △MFI=23), anti-CD40 0.5 μg/ml (· · · · , △MFI=24), anti-CD40 1.0 μg/ml (·····, △MFI=32), anti-CD40 2.0 μg/ml (- - -, △MFI=36), c: IL-4 50 U/ml+anti-CD40 0.2 μg/ml (———, △MFI=288), IL-4 50 U/ml+anti-CD40 0.5 μg/ml (· · · · , △MFI=352), IL-4 50 U/ml+anti-CD40 1.0 μg/ml (·····, △MFI=385), IL-4 50 U/ml+anti-CD40 2.0 μg/ml (- - -, △MFI=441), d: anti-CD40 1.0 μg/ml+IL-4 20 U/ml (———, △MFI=285), anti-CD40 1.0 μg/ml+IL-4 50 U/ml (· · · · , △MFI=410), anti-CD40 1.0 μg/ml+IL-4 100 U/ml (·····, △MFI=491), anti-CD40 1.0 μg/ml+IL-4 200 U/ml (- - -, △MFI=482). Panel B: Graphical presentation of Panel A-a and A-d. Panel C: Graphical presentation of Panel A-b and A-c.

pression, thereby agumenting each other's response.

Role of protein kinases: PTK-dependent and PKC-independent pathways

In order to investigate signaling pathways utilized by IL-4 and anti-CD40 leading to CD23 expression, we have tested effects of various protein kinase inhibitors on the IL-4- or anti-CD40-induced CD23 expression. While IL-4-induced signal transduction pathways for immune cell activation have not been well-defined, our previous studies with small resting human B lymphocytes have shown that although PKC activators are effective inducers of CD23, IL-4 induces CD23 gene expression via pathways independent of PKC (Lee *et al.*, 1993).

As shown in Table 1, in the present study using unfractionated human primary B cells, which is a rather heterogenous population consisting of both resting and

pre-activated B cells, we observed that protein kinase inhibitors, such as staurosporine and H-7, at concentrations effective for selective inhibition of PKC had no effect on the IL-4-induced membrane CD23 expression, which is similar to the results of our study with quiescent B cells. In contrast, protein tyrosine kinase inhibitors genistein and tyrphostin caused a significant suppression (about 50%) of the IL-4-induced CD23 expression on the B cell surface. Interestingly, while anti-CD40 induced surface CD23 expression at a much lower level than by IL-4, effects of protein kinase inhibitors on the anti-CD40-induced CD23 were similar to those on the IL-4-induced response. The selective suppresive effect of tyrosine kinase inhibitors but not PKC inhibitors on the IL-4-induced CD23 expression was also observed at mRNA level (Park *et al.*, 1997). We also have preliminary data demonstrating the inhibitory action of genistein and tyrphostin on the anti-CD40-induced CD23 mRNA (un- published data). It should be noted that there were no cytotoxic effects of these tyrosine kinase inhibitors on B cells at concentrations used in this study. These results indicate that signal transduction of IL-4 and anti-CD40 leading to CD23 expression involves two independent pathways both involving protein tyrosine kinases but not PKC.

Table 1. Effects of protein kinase inhibitors on CD23 expression

Treatment	Mean fluorescence intensity (△MFI)[a]
control media	12.4
IL-4 100 U/ml	116.3
+staurosporine 10 nM	127.1
+H-7 30 μM	114.8
+genistein 100 μg/ml	45.8
+tyrphostin 50 μg/ml	21.1
+steroid 10 μM	90.6
anti-CD40 2 μg/ml	22.6
+staurosporine 10 nM	22.8
+H-7 30 μM	22.3
+genistein 100 μg/ml	11.2
+tyrphostin 50 μg/ml	16.2
+steroid 10 μM	19.3

The purified B cells were pretreated with protein kinase inhibitors for 1 h, washed, and cultured in the fresh media for additional 24 h in the presence of IL-4 or anti-CD40, after which surface CD23 expression was analyzed by flow cytometry as in Materials and Methods.

[a]△MFI (△mean fluorescence intensity)=fluorescence intensity of anti-CD23-FITC-stained cells−fluorescence intensity of control mouse IgG-stained cells. Each value represents a mean of three independent determinations and S.D. is less than 10% of the mean.

IL-4 and anti-CD40 activate distinct nuclear factors binding to the CD23 promoter

As our previous study suggested a presence of IL-4-responsive element (IL-4 RE) near to, but at a distinct site from PKC-responsive element on the human CD23 promoter (Lee *et al.*, 1993), Köhler and Rieber (1993) mapped an IL-4RE (-224 -215) next to the AP1 site (-210~-200) of the human CD23b promoter using the luciferase gene expression as a reporter assay system in human B lymphoblastoid cell line. Thus we have utilized the IL-4RE sequence in EMSA in order to detect nuclear factors involved in the IL-4-induced signal transduction. As shown in Fig. 4, the nuclear extract prepared from B cells treated with IL-4 for 20 min contained a protein factor binding to the IL-4RE of CD23b promoter in a sequence-specific manner (lane 2 and 5). This factor is referred as NF-IL-4, the IL-4-activated nuclear factor conferring IL-4 response by interacting with IL-4RE sequence. Such IL-4RE-binding activity was not induced by anti-CD40 treatment (lane 4), nor the IL-4-induced activity was affected by the co-treatment with anti-CD40 (lane 3), indicating that the IL-4RE is not the target sequence of anti-CD40-induced nuclear factor. The activation of NF-IL-4 was completely blocked by the pretreatment of tyrosine kinase inhibitors but not by PKC inhibitors (lanes 8~11). The result correlates well with the data in Table 1, suggesting that IL-4-induced activation of NF-IL-4 is involved in the CD23 gene expression via PTK-dependent and PKC-independent signaling pathways.

Recently extensive investigations on the CD40-mediated signaling process have been conducted, which revealed the involvement of tyrosine kinases and multiple unidentified serine/threonine kinases (Uckun *et al.*, 1991). For example CD40 engagement by anti-CD40 is shown to induce activation of tyrosine kinase lyn, PLCγ2, PI3-kinase (Ren *et al.*, 1994) ERK, JNK (c-jun N-terminal kinase) (Li *et al.*, 1996) and NF-$_{\kappa}$B (nuclear factor $_{\kappa}$B) (Berberich *et al.*, 1994; Rothe *et al.*, 1995). Since a NF-$_{\kappa}$B binding sequence is found in the CD23 promoter near to the NF-IL-4 site, we were interested in examining whether IL-4 or anti-CD40 stimulation can deliver the signal for CD23 gene induction via NF-$_{\kappa}$B activation in human primary B cells. As seen in Fig. 5, anti-CD40 induced a nuclear factor activity binding to the NF-$_{\kappa}$B site of CD23 promoter (lane 2). However no effect of IL-4 on the NF-$_{\kappa}$B activation or on the anti-CD40-induced NF-$_{\kappa}$B activation was observed (lanes 7 and 9). Pretreatment of cells with tyrosine kinase inhibitors blocked the NF-$_{\kappa}$B activation but a PKC-inhibitor showed no effect (lanes 3~5), which is also consistent with the anti-

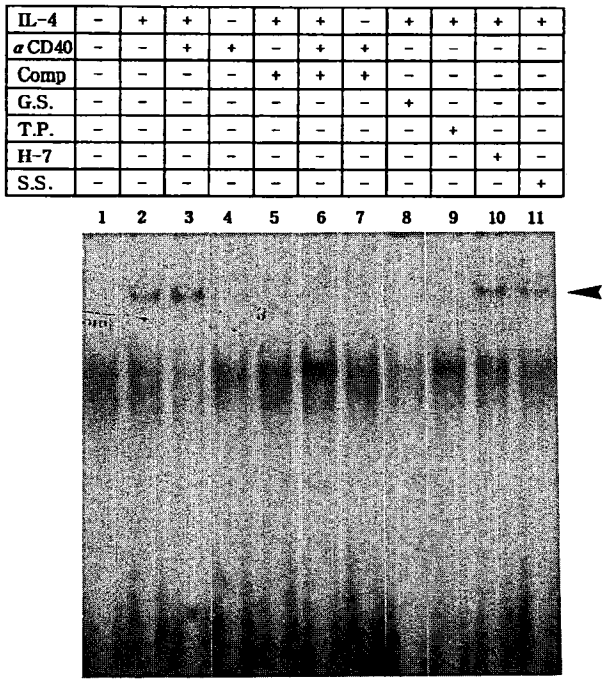

Fig. 4. IL-4, but not anti-CD40, induces activation of a nuclear factor binding to the IL-4RE GAS of CD23b promoter. B cells were treated with IL-4, anti-CD40 or IL-4 plus and anti-CD40 for 20 min, after which nuclear extracts were prepared, and EMSA was performed as in Materials and Methods using IL-4RE GAS sequence (5'GGGTGAATTCTAAGAAAGGG3') of CD23b promoter as a probe. Protein kinase inhibitors [tyrophostin (T.P.) 50 μg/ml, genistein(G.S.) 100 μg/ml, H-7 30 μM and staurosphorine (S.S.) 10 nM] were treated to cells for 30 min prior to the IL-4 addition as indicated. Unlabelled IL-4RE GAS oligomers were used as competitors where indicated.

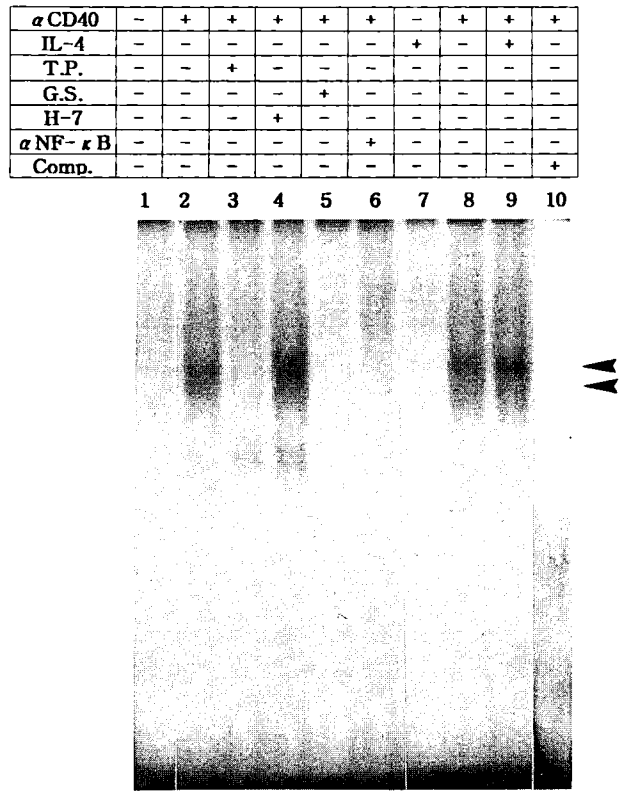

Fig. 5. Anti-CD40, but not IL-4, induces activation of a nuclear factor binding to NF-$_\kappa$B site of CD23b promoter. Cell treatment, nuclear extract preparation and EMSA were performed as in Fig. 4, except that a NF-$_\kappa$B binding sequence (5'GCGGGGCTTCCCAGTC3') found in the CD23b promoter was used as a probe. Unlabelled NF-$_\kappa$B oligomers and polyclonal anti-NF-$_\kappa$B antibody were used to confirm the specificity of binding.

CD40-induced CD23 expression shown in Table 1. Considering the suggested role of PKC in the activation of NF-$_\kappa$B through I$_\kappa$B phosphorylation, displacement from NF-$_\kappa$B complex, and subsequent degradation of I$_\kappa$B, upon various mitogenic or extracellular stimuli in many cell types (Beg *et al.*, 1993; Brown *et al.*, 1995), it is rather interesting to note that anti-CD40-induced NF-$_\kappa$B activation process in human B cells is not affected by PKC inhibitors, and elucidation of the mechanism of such regulation awaits further investigation.

In summary, data presented in this report strongly suggest that distinct nuclear factors, such as NF-IL-4 and NF-$_\kappa$B, are involved in the IL-4 and anti-CD40-induced CD23 gene expression, respectively, and activation of each factor is separately regulated via PTK-dependent and PKC-independent signaling pathways. The utilization of distinct nuclear factors regulating CD23 gene expression would provide a potential mechanism for synergy observed between the action of IL-4 and anti-CD40 as seen in Fig. 2 and Fig. 3. Identification of specific tyrosine kinases participating in each signaling pathways would reveal the exact subcellular location at which two different transmembrane signals converge to exert synergistic effects on the B cell activation and differentiation such as CD23 expression and Ig isotype switching to IgE, and provide further insight into the regulatory mechanism of T cell dependent-B cell activation through soluble as well as membrane-contact signals between two interacting immune cells.

Acknowledgements

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