Akt-dependent Cytokine Production in Mast Cells

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Abstract

Cross-linking of FcεRI induces the activation of three protein tyrosine kinases, Lyn, Syk, and Bruton’s tyrosine kinase (Btk), leading to the secretion of a panel of proinflammatory mediators from mast cells. This study showed phosphorylation at Ser-473 and enzymatic activation of Akt/protein kinase B, the crucial survival kinase, upon FcεRI stimulation in mouse mast cells. Phosphorylation of Akt is regulated positively by Btk and Syk and negatively by Lyn. Akt in turn can regulate positively the transcriptional activity of interleukin (IL)-2 and tumor necrosis factor (TNF)-α promoters. Transcription from the nuclear factor κB (NF-κB), nuclear factor of activated T cells (NF-AT), and activator protein 1 (AP-1) sites within these promoters is under the control of Akt activity. Accordingly, the signaling pathway involving IκB-α, a cytoplasmic protein that binds NF-κB and inhibits its nuclear translocation, appears to be regulated by Akt in mast cells. Catalytic activity of glycogen synthase kinase (GSK)-3β, a serine/threonine kinase that phosphorylates NF-AT and promotes its nuclear export, seems to be inhibited by Akt. Importantly, Akt regulates the production and secretion of IL-2 and TNF-α in FcεRI-stimulated mast cells. Altogether, these results revealed a novel function of Akt in transcriptional activation of cytokine genes via NF-κB, NF-AT, and AP-1 that contributes to the production of cytokines.

Key words: FcεRI • Lyn • Btk • NF-κB • signal transduction

Introduction

Akt/protein kinase B (referred to as Akt hereafter), originally identified by its similarity to protein kinases A and C (1, 2) and also found in a rodent oncogenic retroviral genome (3), is a pleiotropic protein serine/threonine kinase composed of an NH2-terminal pleckstrin homology (PH) domain and a COOH-terminal catalytic domain. Akt is activated by numerous stimuli and is implicated in a variety of cellular functions, such as survival, metabolism, transcription, and translation (for reviews, see references 4–9). In response to growth factors that activate phosphatidylinositol 3-kinase (PI3K), the membrane-bound lipid phosphatidylinositol 3,4,5-trisphosphate (PIP3) is synthesized and recruits 3-phosphoinositide-dependent kinase (PDK)1 (10–12) by binding to its PH domain. PIP3 and phosphatidylinositol 3,4-bisphosphate recruit Akt to the plasma membrane by interacting with the PH domain of Akt (13, 14). PDK1 phosphorylates Thr-308 of Akt and PDK2, a putative kinase yet to be cloned (10), phosphorylates Ser-473 of Akt. Akt phosphorylated at both sites becomes active and phosphorylates target proteins. Other stimuli such as heat shock, hyperosmolarity, okadaic acid, and cAMP activate Akt in a PI3K-independent manner (15–18).

Mast cells are crucial effector cells for IgE-dependent immediate hypersensitivity (19). Contact with multivalent antigen activates IgE-bound mast cells, culminating in degranulation releasing vasoactive amines and secretion of lipid mediators, various proteases, and various cytokines. This high-affinity IgE receptor (FcεRI)-dependent activa-
tion has been a focus of intense study. FceRI consists of four subunits, i.e., one IgE-binding \( \alpha \) subunit, one \( \beta \), and two S-S-bonded \( \gamma \) subunits (20). According to the broadly accepted hypothesis (21–23), FceRI cross-linking elicits the activation of \( \beta \) subunit-bound Lyn, a Src family protein tyrosine kinase (PTK). Active Lyn phosphorylates tyrosine residues in the immunoreceptor tyrosine-based activation motifs (ITAMs [24]) within both \( \beta \) and \( \gamma \) subunits. Phosphorylated \( \beta \)-ITAM and phosphorylated \( \gamma \)-ITAM recruit Lyn and Syk, respectively, via Src homology (SH) 2 domain–phosphotyrosine interactions. Lyn has an SH2 domain, and Syk has two tandem SH2 domains. Activation of these PTKs ensues by phosphorylation of tyrosine residues in the activation loop and conformational changes induced by binding to phosphorylated ITAMs (25–27). Active Lyn and Syk phosphorylate numerous target proteins, including phospholipase C (PLC)-\( \gamma \). Phosphorylation and activation of PLC-\( \gamma \) requires the concerted action of Syk and another PTK, Bruton’s tyrosine kinase (Btk [28, 29]). Hydrolysis of phosphatidylinositol 4,5-bisphosphate by PLC generates two second messengers, inositol 1,4,5-trisphosphate (IP3) and diacylglycerol. IP3 recruits Ca\(^{2+}\) from cellular storage sites, and diacylglycerol activates protein kinase C isoforms (30, 31). Optimal degranulation requires Ca\(^{2+}\) and protein kinase C (32).

Downstream of the above early activation events, three major subfamilies of mitogen-activated protein kinases (MAPKs), i.e., extracellular signal–regulated kinases (ERKs [33–36]), c-Jun NH\(_2\)-terminal kinases (JNKs [37, 38]), and p38 (39), are activated upon FceRI cross-linking. In mast cells, ERK1 and ERK2 are under the control of Syk and R as (39), whereas JNK1 and JNK2 are regulated in a Lyn/ Syk/Btk-dependent manner (38, 40). p38 is also regulated by Btk (38). MAPKs regulate various transcription factors. For example, JNK phosphorylates and activates c-Jun, which dimerizes with Fos and binds to activator protein 1 (AP-1) sites in various gene promoters, including those for cytokine genes (for a review, see reference 41). Promoters in cytokine genes such as those coding for IL-2 and TNF-\( \alpha \) contain other cis-elements that are bound by important transcription factors such as nuclear factor of activated T cells (NF-AT) and nuclear factor \( \kappa \)B (NF-\( \kappa \)B [42–46]). Nuclear location and activity of NF-AT are controlled by Ca\(^{2+}\)-dependent phosphatase calcineurin and R as (for a review, see reference 47). NF-\( \kappa \)B is activated by a variety of inflammatory stimuli via the recently defined pathways that involve NF-\( \kappa \)B–inducing kinase (NIK [48, 49]), Cot/ Tpl-2 (50), and M E K kinase (MEKK)I, -2, and -3 (51–54). These MAPK kinase kinases can activate the IкB kinase (IKK) complex of serine/threonine kinases and a co-factor (55–59). IKK in turn phosphorylates and induces the degradation of IкB (an NF-\( \kappa \)B-sequestering protein family; for a review, see reference 60). However, signaling pathways leading to the activation of NF-\( \kappa \)B in mast cells are little known.

In this study, we found thatAkt is activated upon cross-linking of FceRI in mast cells. Using primary cultured mast cells and mast cell lines deficient in PTK critical for mast cell activation, we investigated the requirements for Akt activation. Downstream of Akt activation, the signaling pathways to transcriptional activation of cytokine genes were analyzed. Importantly, we revealed that Akt is critically involved in cytokine production induced by FceRI stimulation.

**Materials and Methods**

Reagents. Culture media and FCSs were purchased from Life Technologies. Sources of commercial antibodies are as follows: anti-Btk (M-138), anti-Lyn (44), anti-Syk (C-20), anti–PLC-\( \gamma \)2 (Q-20), anti–JNK1 (C-17), and anti–ERK1 (C-16) were from Santa Cruz Biotechnology, Inc.; antiphosphotyrosine mAb 4G10, anti–PLC-\( \gamma \)1 mAbs and anti-Akt antibody were from Upstate Biotechnology; anti-phospho-Akt (Ser473), antiphospho-Akt (Thr308), antiphospho-1kB–\( \alpha \) (Ser32), and antiphospho-MAPK were from New England Biolabs, Inc.; and antiphospho–glyco- gen synthase kinase (GSK)-3\( \beta \) (Ser9) and anti–GSK-3\( \alpha \)/\( \beta \) were from Biosource International. A PTK inhibitor (genistein), PI3K inhibitors (wortmannin and LY 294002), and Pansorbin were purchased from Calbiochem. Other chemicals of the highest grade available were obtained from Sigma-Aldrich or Fisher Scientific, unless otherwise mentioned.

Cells. Btk\(^{\text{+/+}}\) and lyn\(^{\text{+/+}}\) mice, each on a mixed C57BL/6 x 129/Sv genetic background, were mated to generate btk\(^{\text{+/+}}\)/lyn\(^{\text{+/+}}\) F1 progeny. These F1 mice were mated to obtain wild-type (wt), btk\(^{\text{−/−}}\) lyn\(^{\text{−/−}}\), and btk\(^{\text{−/−}}\) lyn\(^{\text{−/−}}\) mice (61). Genotyping was done by Southern blotting or PCR analysis of mouse tail–derived DNAs. Mast cells were cultured as described previously (62). In brief, bone marrow cells derived from femur of the 6–10 wk-old mice were cultured in RPMI 1640 medium supplemented with 10% FCS, 100 \( \mu \)M nonessential amino acids, 50 \( \mu \)M 2-ME, and 8% conditioned medium of IL-3 gene-transfected cells (bone marrow–derived mast cell [BM M C] medium). More than 95% of the Trypan blue–excluding viable cells were mast cells after 4 wk of culture. No discernible differences in morphology and expression of early signaling proteins, including FceRI/\( \beta \), FceRI/\( \gamma \), Syk, Grb2, PLC-\( \gamma \)2, c-Cbl, and Shc were detected among these four types of BM M Cs (data not shown). Surface expression of FceRI at similar levels was confirmed by flow cytometry using a FACS–Calibur apparatus and C E L L Q u o t e t m 8 software (Becton Dickinson).

In acute (\( \sim \)60 min) FceRI signal stimulation experiments, BM M Cs were sensitized by an overnight incubation with 0.5–1 \( \mu \)g/ml anti-dinitrophenyl (DNP) IgE mAb, washed once in Tyrode buffer (112 mM NaCl, 2.7 mM KCl, 0.4 mM NaH\(_2\)PO\(_4\), 1.6 mM CaCl\(_2\), 1 mM MgCl\(_2\), 10 mM Hepes [pH 7.5], 0.05% gelatin, 0.1% glucose), resuspended in Tyrode buffer to \( \times 10^7 \) cells/ml, and stimulated by polyvalent antigen, 100 ng/ml DNP conjugates of human serum albumin (DNP-HSA) for the indicated time intervals.

Rat basophilic leukemia (RBL-2H3) is a rat mast cell line used extensively for studies on FceRI signal transduction. A Syk-deficient RBL-2H3 variant and its syk cDNA-transfected cell line (63) were provided by Dr. R euben P. Straganian (N ational Institutes of Health, Bethesda, M D). A retroviral transfection. H emagglutinin (HA)-tagged Akt cDNAs were recloned into the EcoRI and X hoI sites of the retroviral vector pMX-puro (38). These plasmids were transfected into BO SC-23 packaging cells with Lipofectamine (Life Technolo-

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brene, and drug selection was started 48 h after infection. Mouse populations of puromycin-resistant cells were grown and then cultured in the absence of selection drug for 48 h before FcεRI stimulation.

Measurements of Secreted Cytokines. For cytokine measurements, mast cells were stimulated in BM MC medium instead of Tyrode buffer. TNF-α and IL-2 secreted into the culture medium for 20 h were measured by ELISA kits (Endogen).

Immunoblotting and Immunoprecipitation. Cells were lysed in ice-cold 1% NP-40-containing lysis buffer (20 mM Tris·HCl [pH 8.0], 0.15 M NaCl, 1 mM EDTA, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 25 μg/ml p-nitrophenyl p'-guanidinobenzoate, 1 μM pepstatin, and 0.1% sodium azide) immediately after stimulation. Lysates were centrifuged in an Eppendorf microcentrifuge at 4°C for 10 min. Protein concentrations were measured using DC protein assay reagents (Bio-Rad Laboratories). Immune complexes were washed in lysis buffer four times before SDS-PAGE analysis. For immunoprecipitation, lysates were incubated on ice with an appropriate antibody for 2–4 h, and immune complexes were recovered by brief centrifugation after another 30-min incubation with Pansorbin for rabbit polyclonal antibodies or anti–mouse immunoglobulin-conjugated agarose (Sigma-Aldrich) for mouse mAbs. Lysates were either directly analyzed by SDS-PAGE or immunoprecipitated before SDS-PAGE analysis. Proteins separated by SDS-PAGE were electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad Laboratories). Membranes were blocked, then incubated consecutively with primary antibody and horseradish peroxidase–conjugated secondary antibody, and immunoreactive proteins were visualized by enhanced chemiluminescence reagents (NEL Life Science Products).

Immune Complex Kinase Assays. Akt kinase assays were performed as described previously (64). In brief, cells were lysed on ice for 15 min in ice-cold 1% NP-40-containing lysis buffer. Cleared lysates were incubated with anti-Akt (Upstate Biotechnology) and protein G agarose (Santa Cruz Biotechnology, Inc.). Akt immunoprecipitates were washed four times in the lysis buffer and once with kinase buffer (20 mM 3-[N-morpholino]propanesulfonic acid [MOPS], pH 7.2, 25 mM sodium β-glycerophosphate, 1 mM dithiothreitol [DTT], 15 mM MgCl2, 5 mM EGTA, and 1 mM Na orthovanadate). Washed immunoprecipitates were incubated at 30°C for 15 min with kinase buffer supplemented with 1 μg/ml microcystin (Sigma-Aldrich) and 2.5 μg histone H2B in the presence of [γ-32P]ATP. Reactions were terminated with SDS sample buffer and analyzed by SDS-PAGE, followed by electroblotting onto PVDF membranes and autoradiography. ERK and JNK kinase assays were done as described previously (38). Cells were lysed in 1% NP-40-containing lysis buffer. Cleared lysates were immunoprecipitated with anti-ER K1 (Zymed Laboratories) or JNK1 with an aid of Pansorbin. Immunoprecipitates were washed three times with 1% NP-40-containing lysis buffer and once with kinase buffer (20 mM Hepes [pH 7.4], 10 mM MgCl2, 22 mM DTT, 20 mM β-glycerophosphate, and 50 μM Na3VO4). Immune complexes were incubated with 3 μg myelin basic protein (for ERK assays) or 3 μg of glutathione S-transferase (GST)-c-Jun(1–79) (for JNK assays) in the kinase buffer in the presence of [γ-32P]ATP. Reactions were analyzed as above.

Transcriptional Activity Assay with Luciferase Reporter Constructs. Luciferase reporter constructs, the mouse IL-2 (−321)/luc, the human TNF-α (−200)/luc, NF-κB/luc, AP-1/luc, and NF-AT/luc were described previously (65). 1–1.5 × 106 mast cells were transfected with 5–10 μg reporter plasmids singly or together with 2 or 20 μg each of empty vector, wt Akt (66), E40K Akt, K179M Akt (provided by Dr. P. Blume-Jensen, Sak Institute, La Jolla, CA; reference 67), AAA Akt vectors (provided by Dr. J.R. Moodgett, O tario Cancer Institute, Toronto, Canada; reference 68), dominant negative (DN) IκB-α (1xβαM); provided by Dr. V. Antwerp, Sak Institute, La Jolla, CA; reference 69), DN IKKα (provided by A. Altman, La Jolla Institute for Allergy and Immunology, San Diego, CA; reference 70, or DN GSK-3β (K85M/K86I mutant; provided by J.R. Moodgett and G. R. Crabtree [Stanford University, Palo Alto, CA]; reference 71) by electroporation at 400 V, 950 μF using a Gene Pulser II apparatus (Bio-Rad Laboratories). Both K179 Akt and AAA Akt with K179A/T308A/S473A substitutions are DN mutants. Transfected cells were sensitized overnight with anti-DNP IgE and left unstimulated or stimulated with 30 ng/ml DNP-HSA for 8 h before cell harvest. Cells were lysed in 0.2% Triton X-100 in 100 mM potassium phosphate buffer (pH 7.8)/1 mM DTT. Luminescence of cleared lysates was measured after addition of luciferin solution using a model Monolight 2010 luminometer (Analytical Luminescence Laboratory).

Results

Akt A dition by FcεRI Cross-Linking in Mast Cells. Because Akt is activated by numerous stimuli, we investigated whether FcεRI cross-linking induces Akt activation. In situ Akt activity was monitored before and after cell stimulation by immunoblotting with a phosphospecific antibody to the phosphorylated Ser-473 of Akt. Ser-473 phosphorylation is

Figure 1. Activation of Akt in mast cells by growth factor or FcεRI stimulation. (A) BM MCs sensitized overnight with anti-DNP IgE were stimulated with 100 ng/ml DNP-HSA for the indicated amounts of time. Lysates were analyzed by SDS-PAGE followed by electroblotting onto PVDF membranes and autoradiography. Both K179 Akt and AAA Akt with K179A/T308A/S473A substitutions are DN mutants. Transfected cells were sensitized overnight with anti-DNP IgE and left unstimulated or stimulated with 30 ng/ml DNP-HSA for 8 h before cell harvest. Cells were lysed in 0.2% Triton X-100 in 100 mM potassium phosphate buffer (pH 7.8)/1 mM DTT. Luminescence of cleared lysates was measured after addition of luciferin solution using a model Monolight 2010 luminometer (Analytical Luminescence Laboratory).
crucial for Akt activation. Antigen treatment of IgE-primed BM M Cs caused a remarkable phosphorylation of Ser-473 at its peak ~3–10 min after antigen stimulation (Fig. 1 A, left). Enzymatic activation of Akt in a time course similar to that of Ser-473 phosphorylation was shown in vitro kinase assays on anti-Akt immunoprecipitates using histone H2B as an exogenous substrate (Fig. 1 A, right). Dependence of FcεRI-induced Akt activation on PTK was revealed by pretreatment of BM M Cs with genistein, similar to BCR-induced Akt activation (64, 72, 73). PI3K inhibitors wortmannin and LY294002 also blocked Akt activation very efficiently, confirming the PI3K dependence of Akt activation (Fig. 1 B). These results are consistent with the presence of active mutations of c-Kit, the receptor for stem cell factor (SCF), in this and other mast cell lines (78). But basal and induced levels of Akt phosphorylation were significantly reduced in Syk-deficient RBL-2H3 cells and enhanced in Syk-deficient cells transfected with wt syk cDNA (Fig. 2 C). These results demonstrate that Akt activity is regulated positively by Syk and Btk and negatively by Lyn in mast cells.

Regulation of IL-2 and TNF-α Promoters by Akt. One of the cardinal features of FcεRI-induced mast cell activation is the production and secretion of various cytokines including IL-2 and TNF-α, at least partly through the transcriptional activation of these cytokine genes. Our recent studies indicate that this aspect of mast cell activation is exaggerated in lyn−/− cells (79), and Btk is required for optimal production of these cytokines (80). Given the Akt hyperphosphorylation in lyn−/− cells and the Akt hypophosphorylation in btk−/− cells, we examined whether Akt activity is involved in cytokine gene expression. To this end, we cotransfected BM M Cs with wt or DN Akt cDNA expression vectors together with IL-2/luc or TNF-α/luc reporter plasmids (Fig. 3). Overexpression of wt Akt enhanced twofold induction of IL-2 promoter-driven luciferase expression over the vector-transfected cells upon FcεRI cross-linking. FcεRI-induced transcriptional activation was almost abrogated by two different DN Akt mutants. Similar results were obtained with TNF-α/luc. Given these results, together with higher transcriptional activity of IL-2 and TNF-α promoters (Figure 8 in reference 79) and higher production of these cytokines (Figure 7 in reference 79) in lyn−/− mast cells compared with wt

![Figure 2. PTK dependence on Akt activation induced by FcεRI cross-linking. (A) BMMCs derived from wt, lyn−/−, and btk−/−lyn−/− mice were sensitized by IgE and stimulated with antigen for the indicated amounts of time. Cells were analyzed for Akt Ser-473 phosphorylation and Akt amounts. Stim., stimulation. (B) wt and btk−/− BMMCs and (C) Syk-deficient (syk−) and Syk-reconstituted (syk+) Syk-deficient RBL-2H3 cells were similarly analyzed. Similar results shown in A–C were reproduced in two more independent experiments. Stim., stimulation.](image)

![Figure 3. Akt regulation of IL-2 and TNF-α promoter activities. wt BM M Cs were transfected with IL-2/luc or TNF-α/luc reporter plasmids together with an empty vector or a vector coding for wt, K179M, or AAA Akt. 24 h later, cells were sensitized with IgE overnight. Cells were then stimulated with antigen for the last 8 h before luciferase assays. The IL-2/luc results are representative of three transfection experiments, and the TNF-α/luc results are representative of two experiments. Stim., stimulation.](image)
cells, higher cytokine production in FcεRI-stimulated lyn−/− cells may be accounted for at least partly by the higher activation levels of Akt via the transcriptional regulation. Indeed, overexpression of wt Akt in lyn−/− cells enhanced the transcriptional activity of IL-2 and TNF-α promoters more than that in wt cells, and DN Akt inhibited it (data not shown).

Transcription Factor NF-κB Is under the Control of Akt. Expression of IL-2 and TNF-α genes requires several transcription factors, including NF-κB, AP-1, and NF-AT (65). Therefore, we examined whether the activity of these transcription factors is regulated by Akt in mast cells. First, the luciferase gene under the control of multiple copies of the NF-κB site (−206 to −202, eight times) of the murine IL-2 promoter was cotransfected into BM MCs with wt, E40K, or DN Akt (Fig. 4 A). wt Akt transfection doubled NF-κB-dependent transcription before stimulation, whereas FcεRI stimulation did not enhance it any further. Constitutively active E40K mutant transfectants exhibited a further enhancement in basal and induced levels of NF-κB activity. Importantly, these activities were strongly inhibited by DN Akt. Because of the possible general negative effect of DN Akt expression, we evaluated whether DN Akt induces apoptosis in BM MCs. Because the efficiency of transient transfection used in the above experiments was too low (<20%), to address this issue, we made stable transfectants using wt or DN Akt retroviral constructs. Puromycin-resistant transfectants were stained with annexin V (to detect early apoptotic cells) and propidium iodide (to detect late apoptotic or dead cells), followed by FACS® analysis. The results indicated no enhanced apoptosis in DN Akt-transfected cells (data not shown; see Fig. 6 A for Akt expression). Therefore, these results indicate that Akt positively regulates the NF-κB activity.

IkB regulates NF-κB by sequestering NF-κB in the cytoplasm, and phosphorylation by IKK and subsequent degradation of IkB releases this inhibition (60). Therefore, we examined whether wt IkB-α (IkBaM) and DN IKKα (K44M) affect NF-κB/luc reporter activity. As shown in Fig. 4 B, transcriptional activation of NF-κB was strongly inhibited by DN IkB-α or DN IKKα. In keeping with these results and the enhanced Akt phosphorylation in lyn−/− cells, phosphorylation of Ser-32 in IkB-α (by IKK) was severalfold higher in FcεRI-stimulated lyn−/− mast cells than that in wt cells (Fig. 4 C). These data are also consistent with a recent study that demonstrated that Akt can directly phosphorylate IKKα at Ser-32 (81).

Transcription Factors NF-AT and AP-1 Are also Regulated by Akt. Luciferase constructs driven by multiple copies of other cis-elements of the IL-2 promoter, i.e., NF-AT (−290 to −261, seven times) and AP-1 (65), were also tested by cotransfection with wt or DN Akt (Fig. 5 A and B). NF-AT/luc activity was significantly enhanced in FcεRI-stimulated, wt Akt-transfected cells compared with FcεRI-stimulated, vector-transfected cells. Importantly, these activities were inhibited by two DN Akt (K179M and AAA) mutants, indicating a role for Akt in signal transduction leading to activation of this transcription factor in mast cells. AP-1/luc activity was also affected significantly, albeit to a lesser extent, by wt or DN Akt expression.

JNK is involved in the regulation of both NF-AT and AP-1 activities (41, 65). Therefore, effects of Akt on JNK activity were examined with wt BM MCs stably transfected with wt or DN Akt (Fig. 5 C). FcεRI-induced JNK1 activation was not significantly affected by wt or DN Akt. Similar to JNK activity, FcεRI-induced ERK activation, as measured by phosphorylation at the activation-loop Thr-202 and Tyr-204 residues of ERK1 and ERK2, was barely affected by wt and DN Akt.

These data suggest that the effect of Akt on NF-AT and AP-1 activities is not through the regulation of JNK. GSK-3 controls the nuclear export of NF-AT (71). Phosphorylation at Ser-9 of GSK-3β by Akt inhibits its catalytic activity and blocks the nuclear export of NF-AT. Therefore, we
compared the phosphorylation status of Ser-9 of GSK-3β between wt and lyn−/− BMMCs. As shown in Fig. 5 D, Ser-9 phosphorylation in GSK-3β was higher at ~10–30 min after FcεRI stimulation in lyn−/− cells than in wt counterparts. A slower mobility of GSK-3β on SDS gels was observed at ~10–30 min in wt cells, and this mobility shift was more pronounced in lyn−/− cells than in wt cells. These data are consistent with the notion that Akt-mediated NF-AT activation in FcεRI-stimulated mast cells is through the phosphorylation and inhibition of GSK-3 by Akt. To further examine whether GSK-3 is involved in the regulation of NF-AT activity, transient cotransfection with DN GSK-3β and NF-AT/luc plasmids. DN GSK-3β expression enhanced the FcεRI-induced activation of NF-AT-driven transcription (Fig. 5 A). Expression of DN GSK-3β also enhanced the FcεRI-induced activation of AP-1-driven transcription (Fig. 5 B). Given the lack of the effect of Akt on the activity of JNK and ERK, Akt-mediated AP-1 activation may also be through GSK-3, as GSK-3 is known to inhibit AP-1 activity by phosphorylating Jun (82).

Akt regulates cytokine production in mast cells. All the above data suggest that cytokine production may be regulated by Akt through the regulation of multiple transcription factors including NF-κB, NF-AT, and AP-1. To directly test this possibility, we used puromycin-resistant, stable transfectants of wt BMMCs by retroviral infection with empty vector, HA-tagged wt, or K179M Akt viruses. Transfected Akt was expressed at approximately two- to threefold more than the endogenous Akt level (Fig. 6 A). Akt Ser-473 phosphorylation induced by FcεRI stimulation in wt Akt–transfected cells was higher than that in control vector–transfected cells, whereas it was less pronounced in K179M Akt–transfected cells (Fig. 6 A). More than 98% of these transfected cells were viable in culture medium containing IL-3 and SCF. Upon FcεRI stimulation, wt Akt transfectants secreted more IL-2 and TNF-α than vector-transfected cells, whereas K179M Akt transfectants secreted less than vector-transfected cells (Fig. 6 B). lyn−/− BMMCs transfected with wt or K179M Akt viruses exhibited similar patterns of IL-2 and TNF-α production (data not shown). Taken together, these data indicate that Akt positively regulates IL-2 and TNF-α production and secretion in mast cells.

**Discussion**

This study indicates that Akt is activated by FcεRI stimulation in mast cells. Extracellular stimuli that induce Akt activation include growth factors, cytokines, and antigen
receptors. Most, if not all, of these stimuli promote cell survival and proliferation. Indeed, FcεRI cross-linking and stimulation with SCF and IL-3, two major mast cell growth factors, exhibited these properties, i.e., survival/proliferation and Akt activation (Fig. 1; reference 83; data on Akt phosphorylation by IL-3 and SCF not shown). Although mechanisms by which Akt activation contributes to cell proliferation are not fully understood, some of the known Akt targets are involved in cell survival. A proapoptotic member of the Bcl-2 family BAD is phosphorylated at Ser-136 by Akt (67, 84, 85). The phosphorylated BAD becomes sequestered with and inhibit the survival activity of the proteins Bcl-2 or Bcl-XL. However, some cytokine-mediated cell survival and Akt activation are not correlated with BAD phosphorylation (86–88). Caspase-9, an initiator caspase of apoptosis, is another target of Akt, and phosphorylation of caspase-9 at Ser-196 by Akt inhibits its protease activity (89). Another antiapoptotic mechanism by Akt is phosphorylation of forkhead family transcription factors. Phosphorylation of multiple sites in FKHR L1, AFX, and FKHR 1 transcription factors results in inhibiting their transcriptional activity (90–92). FKHR L1 phosphorylated by Akt is bound to 14-3-3 proteins and retained in the cytoplasm, and is prevented from activating the gene(s) involved in apoptosis (90). It remains to be explored whether any of these known antiapoptotic mechanisms contribute to the mast cell survival/proliferation induced by FcεRI stimulation.

A hallmark of FcεRI-induced mast cell activation is the production and secretion of various cytokines including IL-2, IL-2, IL-3, IL-4, IL-6, IL-9, IL-13, GM-CSF, TNF-α, etc. Among this expanding list of cytokines, TNF-α is known to play a critical role in late phase reactions of hypersensitivity (19). Potential mechanisms for allergic inflammation may include the antiapoptotic effect of TNF-α on monocytes (93). Production of cytokines such as IL-2 and TNF-α is regulated at several steps: gene transcription, mRNA stability, translation, and posttranslational modification. One of the critical regulatory steps is transcription. As shown for other cell types, numerous cis-transcriptional elements that are binding sites for transcription factors are involved in transcriptional activation of the IL-2 gene in FcεRI-stimulated mast cells (65). This study provides evidence that Akt regulates transcriptional activity of NF-κB, NF-AT, and AP-1 that is critical for the expression of the IL-2 and TNF-α genes. Phosphorylation of the inhibitor IκB-α seems to be under the control of Akt as a signaling intermediary from Akt to NF-κB, because phosphorylation of IκB-α at Ser-32 (the phosphorylation site by IKK) was enhanced in lyn-/- mast cells in which Akt is hyperactivated. Because DN IκB-α and DN IKK inhibit FcεRI-induced NF-κB activity, Akt may phosphorylate and activate IKK, and IKK in turn phosphorylates and promotes the degradation of IκB. Then free from IκB, NF-κB can translocate to the nucleus. During the revision of this manuscript, two groups showed that Btk regulates NF-κB in B cells (94, 95), in apparent agreement with our results in mast cells. As several other kinases such as Cot, MEKK1-3, and NF-κB-inducing kinase were shown to be capable of phosphorylating IKK, it will be interesting to determine whether Akt directly phosphorylates IKK in mast cells, as shown for TNF- and platelet-derived growth factor (PDGF)-stimulated cells (81, 96).

GSK-3 phosphorylation by Akt may be involved in NF-AT and AP-1 activation in mast cells. Akt can phosphorylate and inhibit GSK-3 (71). Consistent with this established fact, phosphorylation of GSK-3β at Ser-9 was enhanced in lyn-/- mast cells, which exhibit hyperactive Akt upon FcεRI stimulation. GSK-3 in turn is known to phosphorylate NF-AT and regulate the nuclear exit of this transcription factor. GSK-3 is also known to phosphorylateJun proteins to inhibit AP-1 activity (82). Although Akt overexpression exhibited little effect on ERK and JNK activity, it significantly affected the activities of NF-AT and AP-1 (Fig. 5). Therefore, Akt-dependent phosphorylation of GSK-3 may regulate NF-AT and AP-1 without substantially affecting the canonical MAPK activation pathways, i.e., the Raf-1/MEK/ERK and MEKK/MAPK kinase 4 (or MAPK kinase 7)/JNK pathways.

In summary, we provide the first evidence for the involvement of Akt in FcεRI-induced production of IL-2 and TNF-α. Production of these cytokines is critical to late...
enzyme reactions of IgE-dependent hypersensitivity. Although Akt itself may not be a target for pharmaceutical interference to control allergic reactions and immunological diseases, our findings of Akt as a novel component of the signaling pathways leading to cytokine production have provided new insight into the exquisite networks of signaling molecules for mast cell activation.

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