厚生労働科学研究研究費補助金
こころの健康科学研究事業

終板アセチルコリンエステラーゼ欠損症、及び、他の細胞外マトリックス分子欠損症におけるタンパク標的療法の開発研究

平成18年度　総括研究報告書

平成19(2007)年4月

主任研究者　大野　欽司

目　次

I. 終板アセチルコリンエステラーゼ欠損症、及び、
他の細胞外マトリックス分子欠損症におけるタンパク標的療法の開発研究

主任研究者　大野　欽司　名古屋大学医学系研究科・神経遺伝情報学・教授

研究要旨

先天性筋無力症候群は、神経筋接合部の先天的分子欠損症が原因であり、主任研究者らは欠損分子に応じた治療法を開発・臨床応用してきた(Engel, Ohno, Sine. *Nat Rev Neurosci* 4: 339, 2003)。しかし、collagen Q分子(*COLQ*遺伝子産物)欠損による終板アセチルコリンエステラーゼ(AChE)欠損症は全く治療法が存在しない(Ohno, et al. *Proc Natl Acad Sci USA* 95: 9654, 1998)。Collagen Q 3分子(*COLQ*遺伝子産物)は3重鎖を形成し、AChE catalytic subunit 12分子と結合し、非対称性A_{12}-AChEを形成する。本研究では、collagen Qがシナプス基底膜への係留シグナルを有する細胞外構造タンパクであることを利用し(Kimbell*, Ohno*, et al. *J Biol Chem* 279: 10997, 2004. *equal contribution)、*COLQ*欠損モデル動物のリンパ球に正常*COLQ*遺伝子と正常*ACHE*遺伝子を導入し、A_{12}-AChEを血中に発現させ、シナプス基底膜への係留を試みる。一般に、遺伝子治療においては導入遺伝子の細胞特異的・組織特異的なターゲッティングが障壁となり、培養細胞レベルで有効である手法の多くが臨床応用できない。本研究では、組み替え遺伝子を標的組織にターゲッティングさせる代わりに、欠損タンパクが細胞外分子であることと、タンパクに標的組織親和性があることを利用して標的組織へのターゲッティングを行う。本手法は、他の神経難治疾患であるperlecan欠損症、α2 laminin欠損症、α dystroglycanopathyを含む細胞外マトリックスタンパク欠損症一般への応用の可能性がある。また、本研究では、骨格筋に対する親和性のあるadeno-associated virus (AAV) serotype 6にヒト*COLQ*遺伝子を組み込みマウス尾静脈から全身投与を行い、神経筋接合部における非対称性A_{12}-AChEの発現を確認する。

A. 研究目的

神経筋接合部の分子欠損症による先天性筋無力症候群は、世界中から数多くの症例が報告されているが日本からの報告は少ない。諸外国でもde novo遺伝子変異が数多く存在することから、本症候群が日本にだけ少ない可能性は少ない。胎生期からの神経筋接合部伝達障害によるanomaly、筋萎縮、関節拘縮がみられ、諸外国で見られるように日本でも筋ジストロフィー症や先天性筋症と診断をされている例が少なからず存在すると思われる。また、重症筋無力症と診断をされ、不必要な胸腺摘出術や免疫抑制療法を受けている症例も、諸外国同様に日本にも数多くあると思われる。先天性筋無力症候群は欠損分子に応じた治療が可能であるタイプが多く、本症候群の診断、及び新規治療法開発研究は、患者利益につながると期待をされる。先天性筋無力症候群の中でも、*COLQ*遺伝子変異による終板AChE欠損症は、従来、全く治療法が存在しない。本研究では、終板AChE欠損症に対して、臨床応用を近視野に入れたタンパク標的療法の開発研

究を行う。本研究にて開発する手法は、神経難治疾患を含む他の細胞外マトリックスタンパク欠損症への応用の可能性があり、本手法を他の分子へ適用するための基盤的研究も行う。福山型筋ジストロフィーを始めとして細胞外マトリックスタンパク欠損による疾患は数多く、また、dominant negative変異タンパクの発現によるガン細胞の制御など、本手法の多疾患への応用の可能性は高いと思われる。

B. 研究方法

Collagen Q（*COLQ*遺伝子産物）は、コラーゲンドメインを介して3分子が3重鎖構造を形成し、acetylcholinesterase (AChE) catalytic subunit（*ACHE*遺伝子産物）12分子と結合し、非対称性A_{12}-AChEを形成する。本研究では、collagen Qがシナプス基底膜への係留シグナルを有する細胞外構造タンパクであることを利用し、*COLQ*欠損モデル動物のTリンパ球に正常*COLQ*遺伝子と正常*ACHE*遺伝子を導入し、A_{12}-AChEを血中に発現させ、シナプス基底膜への係留を試みる。また、本手法の他の細胞外マトリックスタンパク欠損症への応用の可能性を探る。

I. *ColQ*欠損マウスの評価

ヘテロの*ColQ*欠損マウス（フランスINSERMのDr. Eric Krejci より譲渡）を交配し、そこから産まれた*ColQ*+/+, *ColQ*+/-, *ColQ*-/-マウスの表現型について評価を行う。運動機能を調べるため、ロタ・ロッド試験を行う。4分間で0～40 rpmまで加速回転する棒上で、マウスが乗り続けられる時間を計測する。また、*ColQ* +/+, *ColQ* +/-, *ColQ* -/-マウスの筋肉組織中のAChEの分子形状をショ糖濃度勾配遠心法を用いて調べる。

II. レトロウィルスベクターの構築

レトロウィルスベクターを用いて、モデルマウスにヒト*COLQ*遺伝子を導入、発現させる手法の確立を試みる。レトロウィルスベクターpSIREN-RetroQ (Clontech社)に、リンパ球系細胞で活性が高いEF1αプロモーターを組み込み、その下流にヒト*ACHE* cDNA・IRES・ヒト*COLQ* cDNAを組み込む。*COLQ*欠損モデル動物のTリンパ球に遺伝子導入を行う。目的組織へのターゲッティングはColQがシナプス基底膜への係留シグナルを有する細胞外構造タンパクであることを利用する。正常ヒト*ACHE*遺伝子と正常ヒト*COLQ*遺伝子を導入する。このレトロウィルスをパッケージング細胞PLAT-Eに導入し、産出させた組み換えレトロウィルスをNIH 3T3細胞に感染させ、A_{12}-AChEの細胞内発現及び細胞外放出を確認する。

III. マウスリンパ球へのレトロウィルスの導入及びマウスへの注入

正常マウスの脾臓、リンパ節よりCD3及びCD28抗体を用いてTリンパ球を単離する。IL2の存在下に培養を行い、上記レトロウィルスを感染させる。次に、細胞培養液中の組み替えA_{12}-AChEをヘパリンアガロースカラムで精製を行い、ショ糖濃度勾配遠心法により分離し、形質転換Tリンパ球が、組み替えA_{12}-AChEを合成し培養液中に放出することを確認する。

その後、形質転換Tリンパ球を正常マウスに注入する。ヒトAChEに対するモノクローナル抗体(米国Mayo Clinic, Dr. Andrew G. Engelより供与)を用いて、組み換えA_{12}-AChEの血中での発現を調べ、さらに、シナプス基底膜への係留の有無を調べる。次に、形質転換Tリンパ球を*ColQ*-/-マウスに適用し、非対象性A_{12}-AChE分子の発現、及び、神経接合部でのA_{12}-AChE分子の集積、生理学的・形態学的検査を行い、筋無力症状の機能回復・安全性について検討する。

IV. AAV (adeno-associated virus)を用いた*COLQ*遺伝子導入

AAVベクターは、神経・筋・肝細胞等の非分裂細胞に対し一回の導入で長期的な遺伝子発現が得られ、また安全性の点でも非病原性・低免疫原性など他のウイルスベクターに比べ利点が多い。ここでは筋肉組織への親和性が高いserotype 6を選び用いる。AAVベクターは、pAAV-CMV-MCS (Stratagene社)を用いて、骨格筋において高活性を示すCMV promoterの下流に*COLQ*を挿入したコンストラクトを作製する。AAVベクターをヘルパーベクターpDF6と共にリン酸カルシウム法によりHEK293T細胞にトランスフェクションし、得られたrAAVウィルス粒子をAAVHT1080に感染させ、GFP発現細胞をフローサイトメトリーを用いて測定し、細胞への遺伝子導入効率を調べる。

C. 研究結果

I. *ColQ*欠損マウスの評価

ColQ+/-マウスを交配し、そこから産まれた仔の遺伝子型をPCRにより決定した。*ColQ* +/+, *ColQ*+/-, *ColQ*-/-マウスの表現型を調べるため、生後1週間から10週間までの体重を測定した。その結果、*ColQ* +/+, *ColQ*+/-マウスに差はなく、*ColQ*-/-マウスは同じ週令の*ColQ* +/+や*ColQ*+/-個体に比べ約50%の体重しかなかった。さらに、*ColQ*-/-マウスは生後まもなくから持続性の全身震戦がみられ、4週ごろから不随意な発声と寡動および易疲労性を認めた。また、8匹中2匹は4週令までに死亡した。

運動機能を調べるロタ・ロッド試験にて、生後8～9週の運動持続時間を測定した結果、*ColQ*-/-マウスは*ColQ*+/+,や*ColQ*+/-個体に比べ有意に筋力・持久力が低いことが確認され、筋無力症状の評価手法として有用であると思われる。

モデルマウスの筋肉組織中のAChE分子をショ糖密度勾配遠心法による分離した。25%～7%のショ糖溶液により遠心チューブの下から上へショ糖の密度勾配を作り、ホモジナイザーにより抽出したマウスの下肢骨格筋を重層した。同時に沈降マーカーとしてアルカリホスファターゼ(ALPhos)とβ-ガラクトシダーゼ(β-gal)を加えた。これを38,000 x *g*, 21時間超遠心することで、AChEを分画した。遠心後、チューブの下から翼状針で穴を開け80から90のフラクションを取り、これらをそれぞれAChE, ALPhos, β-galの活性検出液に加え、吸光度計により活性を検出した。その結果、*ColQ* +/+マウスでは非対称性A_{12}-, A_8-, A_4-AChEの活性が認められたが、*ColQ*-/-マウスの骨格筋にはColQを欠く球状G_1-, G_2-, G_4-AChEの活性しか認められず、*ColQ*の欠損により非対称性AChEが作られていないことが示された。

II. レトロウィルスベクターの構築

siRNA導入目的に開発されたClontech社のpSIREN-RetroQベクターからU6プロモーターを除去し、Tリンパ球において活性が高いEF1αプロモーターを組み込みんだ。EF1αの下流にヒト*ACHE*遺伝子, *COLQ*遺伝子, IRESを導入するため、それぞれの遺伝子に制限酵素配列を付加し、サブクローニングを行った。遺伝子を一つずつ挿入していき、p-SIREN-EF1α-*ACHE*-IRES-*COLQ*を構築した。作製したベクターを、PLAT-E細胞にトランスフェクションをし、組換えレトロウィルスを得た。GFP遺伝子を導入したPLAT-E細胞をフローサイトメトリーで測定し、細胞へのトランスフェクション効率は40ー50%であり、NIH3T3細胞への感染は1.6x10^7 PFU/mlと高い効率のウィルスができた。

p-SIREN-EF1α-*ACHE*-IRES-*COLQ*をトランスフェクションした細胞からの抽出タンパクを、ショ糖濃度勾配遠心法で分画しAChEの形状を調べた。p-SIREN-EF1α-*ACHE*をトランスフェクションした細胞では球状G_1-, G_2-, G_4-AChEが検出され、p-SIREN-EF1α-*ACHE*-IRES-*COLQ*をトランスフェクションした細胞においては球状AChEに加え非対称性A_{12}-, A_8-, A_4-AChEの活性が検出され、ColQとAChEが共に発現して結合していることが明らかになった。今後、この組換えレトロウィルスを感染させた細胞の培養液を回収し、ヘパリンアガロースカラムで精製を行ないA_{12}-AChEの細胞外放出を確かめる予定である。

III. マウスリンパ球へのレトロウィルスの導入及びマウスへの注入

正常マウスから脾臓、リンパ節を摘出し、血球をCD3及びCD28抗体存在下で培養してTリンパ球の単離を行なっている。現在、Tリンパ球の増殖の良好な培養系の確立を試みている。必要に応じて、anti-CD4抗体磁気ビーズを用いたネガティブセレクションを行ない、純度の高いTリンパ球を得る手法を考えている。Tリンパ球を単離した後は上記組み換えレトロウィルスを感染させ、形質転換Tリンパ球から組み替えA_{12}-AChEが合成され細胞外に移行することを確認する予定である。

IV. AAV (adeno-associated virus)を用いた*COLQ*遺伝子導入

AAVベクターはpAAV-CMV-*COLQ*と、ColQと共にGFPを発現するpAAV-CMV-*COLQ*-IRES-EGFPのコンストラクトを作製した。DNA複合体がエンドサイトーシスにより細胞内に取り込まれると考えられているリン酸カルシウム法では、効率を左右する最も重要な因子はpHである。このため、HBS (HEPES-buffered saline)のpHを7.05、7.07、7.10、7.12、7.16と変えて実験した結果、この中での至適pHは7.16であり、導入効率は20%であった。Real-Time PCR法により、上記の実験で抽出したウィルス液の濃度を測定したところ、0.5～1.7×10^8 (genome copies/ml)となった。

ColQ-/-マウスの尾静脈より全身投与するには、3～4×10^{12} genome copiesの大量のAAVを作製・精製する必要がある。AAVのより効率的な産出方法と精製方法について現在検討を行ってい

る。今後、組換えAAVを*ColQ*-/-マウスの筋肉、または静脈に注入し、骨格筋におけるA_{12}-AChE分子の発現、及び、神経接合部でのA_{12}-AChE分子の集積を調べる。さらに、遺伝子導入を行なった*ColQ*-/-マウスを用い、微小板電位、終板電位、複合筋活動電位などの生理学的検査や、運動機能試験、形態学的検査を行ない、筋無力症状の機能回復を調査する。また、組換えA_{12}-AChEの腎糸球体基底膜などへの異所性集積や抗collagen Q抗体の出現など、遺伝子導入の安全性の検討を今後行う。

D. 考察

昨年度はレトロウィルスの予期せぬ組み換えのためにより、レトロウィルスベクターの構築に予想外に手間取ったが、遺伝子挿入の順序と配列長を検討することにより、目的とするクローンを得ることができた。さらに、ColQ-/-マウスの評価手法を確立し、レトロウィルスを感染させたマウス由来細胞株における非対称性AChEの産生を確認し、次のステップのためのマウスTリンパ球の調整を行った。さらに、今年度より骨格筋親和性のあるAAVベクターを用いた遺伝子治療の検討も開始した。年度途中ではあったが本プロジェクトのリサーチレジデントの着任は研究の大きな推進力になった。

E. 結論

新たな実験手法の確立など今後とも時間を要すると思われる段階が数多くあるが、本プロジェクトは順調に推移してきており、本研究最終年である2007年度に成果を出すべく研究に邁進する。

F. 健康危険情報

特記事項なし。

G. 研究発表

1. 論文発表

1. Masuda A, Hashimoto K, Yokoi T, Doi T, Kodama T, Kume H, Ohno, K, Matsuguchi T. Essential role of GATA transcriptional factors in the activation of mast cells. *J Immunol* 2007, 178:360-368.

2. 学会発表

1 Sahashi K, Shinmi J, Sobue G, Ohno K
How mutations at the 3' end region of exons cause aberrant splicings? - *In silico* and *in vitro* analyses to answer the question -
56th Annual Meeting of the American Society of Human Genetics, New Orleans, Louisiana, USA. Oct 10-14, 2006

2 Kurosaki T, Matsuura T, Ohno K, Ueda S
Comparative analysis of the SCA10 ATTCT pentanucleotide repeat indicates the repeat s

equence originates from retrotransposons

56th Annual Meeting of the American Society of Human Genetics, New Orleans, Louisiana, USA. Oct 10-14, 2006

3 Gao K, Shinmi J, Ohno K

The human branch point sequences are more degenerative than we expect

RNA 2006 "Functional RNAs and Regulatory Machinery", Izu, Shizuoka, Japan Dec 3-7, 2006

H. 知的財産権の出願・登録状況

特記事項なし。

II. わが国における先天性筋無力症候群未診断症例の病態・治療研究

分担研究者　祖父江　元　名古屋大学医学系研究科・神経内科学・教授

研究要旨

先天性筋無力症候群は、神経筋接合部の先天的分子欠損により、顔面・四肢・体幹の筋力低下・筋萎縮・奇形を主徴とする疾患群である。世界中からおよそ200例が報告されており、7種類の神経筋接合部分子において約180種類の遺伝子変異が同定されてきている。これらのうちファウンダー効果が明らかなものは*RAPSN*遺伝子における1変異のみであり、他の多くの遺伝子変異は個々の家系に特有の遺伝子変異か、de novo遺伝子変異であり、人種や地域を問わず本症候群が存在すると想定されるが、わが国からの報告は極めて稀である。本研究では、本症候群の診断に反復神経刺激が有用であることを利用し、筋力低下を主徴とする非定型的な神経筋疾患に対して積極的に反復神経刺激を行い、スローチャンネル症候群の一例を診断した。今回、本例におけるアセチルコリンレセプターチャンネルブロッカーのquinidine 400 mg/dayとfluoxetine 40 mg/dayによる治療効果につき報告する。

A. 研究目的

先天性筋無力症候群は、神経筋接合部の先天的分子欠損症が原因であり、同定されてきた欠損分子には、(1) 神経終末からhigh affinity choline transporterを介して取り込まれたcholineからacetylcholineを再合成するcholine acetyltanferase、(2) acetylcholinesterase catalytic subunitとともにasymmetric acetylcholineを作り、synaptic basal laminaにacetylcholinesteraseをanchoringをさせるcollagen Q、(3) muscle nicotinic acetylcholine receptor (AChR)を形成するAChR subunits、(4) AChRを神経筋終板に集積させるrapsyn、(5) AChRによる脱分極を筋膜全般に伝播するmuscle voltage-gated sodium channel、(6) 神経終末より放出されるneural agrinのレセプターであり、AChRとrapsynの結合を促進し、endplateへのAChRの集積をドライブするMuSK (muscle specific kinase)、(7) MuSKシグナル伝達系の分子でMuSKやrapsynと同じく終板におけるAChR集積に関わるDok-7があげられる。スローチャンネル症候群は、AChRイオンチャンネルの開口時間が延長する病態である。先天性筋無力症候群は、スローチャンネル症候群のみが常染色体優性遺伝であり、他はいずれも常染色体劣性遺伝である。本症候群のなかで、ファウンダー効果が知られている遺伝子変異は、rapsynのN88K変異のみであり、大多数は、個々の家系に特有の変異か、de novo変異である。日本では先天性筋無力症候群と診断された症例は10例以下であり、多くは未診断であったり、誤診のもとに間違った治療を受けている可能性がある。本症候群は重症筋無力症と異なる臨床症状を呈することも診断を困難にしていると思われる。本症候群では、胎生期からの神経筋接合部信号伝達障害があることから顔面・頭蓋骨奇形や骨格筋低形成が認められることがある。また、日内変動が明らかではなく、複視を伴わない眼球運動障害も多く認められる。本症候群の診断には反復神経刺激

が有用であることを利用し、筋力低下を主徴とする非定型的な神経筋疾患に対して積極的に反復神経刺激を行い、スローチャンネル症候群の一例の診断を行なった。AChRαサブユニットに遺伝子変異を同定し、quinidineとfluoxetineによる治療を試みた。

B. 研究方法

筋力低下を主徴とする非定型的な神経筋疾患に対して、低頻度および高頻度反復神経刺激による複合筋活動電位(CMAP)減衰の計測を行い、同時に、単発神経刺激に対する反復CMAPの出現をモニターした。反復CMAPの出現および臨床症状よりスローチャンネル症候群を疑いAChRサブユニット遺伝子変異を同定した。発現実験を行いスローチャンネル症候群であることを確認した。さらに、quinidineとfluoxetineによる治療を試みた。

C. 研究結果

症例は、36歳発症の軽度四肢近位筋力低下と労作性呼吸困難を主訴とする37歳男性である。幼少時からの斜視があるが複視を訴えない。診察時に易疲労性を認めず、日内変動もなく、edrophoniumテストも陰性であり、重症筋無力症を含む神経筋接合部疾患を積極的に疑う臨床所見に欠けている。単発神経刺激にて2発の反復CMAPを認めた。反復CMAPは、(1) スローチャンネル症候群、(2) 終板acetylchoinesterase欠損症、(3) 抗cholinesterase剤や有機リン中毒によるacetylcholinesterase活性抑制で認められる。脛骨神経の3 Hzの反復神経刺激にて22%の異常減衰を認め、 single fiber EMGではMCDが98 ± 54μsと延長しており、神経筋接合部信号伝達の異常が示唆された。

AChRサブユニット遺伝子変異によるスローチャンネル症候群と、collagen Q遺伝子変異による終板acetylcholinesterase欠損症を疑い、網羅的な遺伝子変異の検索を行ったところ、AChR βサブユニットにV296A変異を認めた。患者は正常alleleと変異alleleを持つheterozygoteであり、βV296Aはドミナント変異であった。

βV296は第3膜貫通ドメインほぼ中央部に位置し、αサブユニットで対応するアミノ酸はαV285である。イオンチャンネルの開口時間が異常に短縮するファーストチャンネル症候群においてαV285I変異が報告されている(Wang H-L, Milone M, Ohno K, et al. *Nat Neurosci* 2: 226, 1999)。αV285I変異の研究において、αV285L、αV285I、αV285T、αV285Aの4種類の変異αサブユニットの解析が行われており、コドン285におけるアミノ酸側鎖のボリュームが、イオンチャンネルの動態を決定していることが判明している。つまり、valineよりもボリュームが大きいleucineやisoleucineではファーストチャンネルとなり、valineよりもボリュームが小さいthreonineやalanineではスローチャンネルとなる。この事実は、第3膜貫通ドメインほぼ中央部のコドン285におけるアミノ酸側鎖が、AChRのチャンネル孔を形成する第2膜貫通ドメインを背側から押していることを示している。AChR α, β, δ, εサブユニットは相同なサブユニットであり、第3膜貫通ドメインのβV296Aは第2膜貫通ドメインを背側から押す力を弱め、AChRのチャンネル孔を開きやすくさせるスローチャンネル変異と想定され、事実、単一チャンネル記録にてチャンネル開口が異常に遅延していること

を実験的に証明した。

イオンチャンネルブロッカであり、スローチャンネル症候群に対する有効性が確立しているquinidine常用量(400 mg/day)を投与したところ、四肢筋力が改善し、反復神経刺激に対するCMAPの異常減衰も軽減し、単発神経刺激に対する反復CMAPも軽減した。しかし、寒冷誘発性のアセチルコリン放出効率の低下とAChR開口効率の低下によると思われる寒冷時の四肢筋力低下と呼吸不全を認めたため、fluoxetine常用量(40 mg/day)を追加し、さらなる四肢筋力の改善を認めた。

D. 考察

今回解析を行った症例に見るように先天性筋無力症候群の中には、成人発症で、日内変動を伴わず、易疲労性も明らかでない症例が多く存在すると思われる。今回の症例は呼吸筋が優位に犯されるという重症筋無力症を考えにくい臨床像であり、単発神経刺激による反復CMAPの出現と、反復神経刺激におけるCMAP電位の異常減衰が診断に有用であった。筋力低下を主徴とする神経疾患に対して電気生理学手法を用いた神経筋接合部欠損の検索を行う重要性が示唆された。

E. 結論

先天性筋無力症候群は、スローチャンネル症候群をはじめとして病態に応じた治療方法が可能な疾患群が多く、本症候群に対して積極的に電気生理学診断を行う重要性が示された。

F. 健康危険情報

特記事項なし。

G. 研究発表

1. 論文発表

1. Koike, H, Watanabe H, Inukai A, Iijima M, Mori K, Hattori N, and Sobue G. Myopathy in thiamine deficiency: Analysis of a case *J Neurol Sci* 2006; 249: 175-179.

H. 知的財産権の出願・登録状況

特記事項なし。

III. 研究成果の刊行に関する一覧表

発表者名	論文タイトル名	発表誌名	巻号	ページ	出版年
Masuda A, Hashimoto K, Yokoi T, Doi T, Kodama T, Kume H, Ohno K, Matsuguchi T	Essential role of GATA transcriptional factors in the activation of mast cells	*J Immunol*	178	360-368	2006
Koike, H, Watanabe H, Inukai A, Iijima M, Mori K, Hattori N, and Sobue G	Myopathy in thiamine deficiency: Analysis of a case	*J Neurol Sci*	249	175-179	2006

Essential Role of GATA Transcriptional Factors in the Activation of Mast Cells[1]

Akio Masuda,[2]* Katsunori Hashimoto,‡ Toyoharu Yokoi,‡ Takeshi Doi,§ Tatsuhiko Kodama,¶ Hiroaki Kume,† Kinji Ohno,* and Tetsuya Matsuguchi‖

Mast cells are pivotal effector cells in IgE-mediated allergic reactions. GATA transcriptional factors such as GATA-1 and GATA-2 are expressed in mast cells, and recent studies have revealed that both GATA-1 and GATA-2 are required for mast cell development. However, the role of GATA transcriptional factors in differentiated mast cells has remained largely unknown. In this study, we repressed the activity of GATA-1 and GATA-2 by using three different approaches (inducible overexpression of a dominant-negative form of GATA, pharmacological inactivation, or small interfering RNA technology), and analyzed the molecular mechanisms of GATA transcriptional factors in the activation of mast cells. Surprisingly, the repression of GATA activity in differentiated mast cells led to the impairment of cell survival, IgE-induced degranulation, and cytokine production. Signal transduction and histone modification in the chromatin related to protein kinase Cβ were defective in these cells. These results identify that GATA has a critical role in the activation of mast cell. *The Journal of Immunology,* **2007, 178: 360–368.**

Inflammatory substances released by mast cells induce and maintain the allergic response (1). The major mechanism of the stimulation of these cells is the interaction of Ags with IgE bound to the high-affinity receptor, FcεRI, on the cell surface. This interaction results in the release of preformed mediators from granules and the generation of newly synthesized mediators, such as cytokines and the products of arachidonic acid.

GATA proteins are tissue-restricted transcription factors that bind a WGATAR DNA motif through a zinc-finger DNA-binding domain. Based on sequence homology and expression patterns, GATA proteins have been divided into two subfamilies, i.e., GATA-1–3 and GATA-4–6 (2). The former family is prominently expressed in hemopoietic stem cells and the latter is expressed in various mesoderm- and endoderm-derived tissues (3). Gain-of-function and loss-of-function studies demonstrated the necessity of GATA-1 and GATA-2 for proper hemopoietic development (4).

Mast cells express GATA-1 and GATA-2, and recent studies have revealed that both GATA-1 and GATA-2 are required for the differentiation of mast cells (5–7). GATA-2, rather than GATA-1, appears to regulate early mast cell gene expression because GATA-2 is expressed at higher levels in immature mast cells (8). However, the role of GATAs may not be limited to the differentiation of mast cells, because GATA-1 and GATA-2 are also expressed in mature mast cells that reside in tissues (8, 9). Indirect evidence for a functional role for mast cells is provided by the presence of GATA consensus sequences in the promoter region of *carboxypeptidase A*, the α- and β-chain of the human IgE receptor (*FcεRα* and *β*), *IL-4*, and *IL-13* (10–14). Ectopic GATA-1 or GATA-2 expression activates the promoter activity of these genes. However, because GATA low-activity mutants are either embryonically lethal or are defective with respect to the differentiation of mast cell lineage (6, 7, 15), the physiological role of GATAs in differentiated mast cells remains largely unknown.

In this study, we analyzed the role of GATA transcriptional factors in differentiated mast cells and found that repression of GATA activity leads to the impairment of cell survival, IgE-induced degranulation and cytokine production, and to defective signal transduction. In addition, the repression of GATA activity significantly inhibited anaphylactic responses in vivo. The decreased protein kinase C (PKC)[3] β expression and the down-regulation of histone acetylation at the PKCβ regions were observed in GATA-repressed cells. These results indicated that GATA proteins have a critical role in mast cell activation.

Materials and Methods

Reagents and Abs

Recombinant mouse IL-3 and mouse stem cell factor (SCF) were purchased from PeproTech. Doxycycline (Dox), G418, and hygromycin B were purchased from Wako Pure Chemical. K-7174 was obtained from Kowa. The mouse monoclonal anti-dinitrophenol (DNP) Ab, the DNP-human serum albumin (DNP-HSA), ionomycin, and PMA were purchased from Sigma-Aldrich. Rat monoclonal anti-GATA-1, the polyclonal anti-GATA-2, the polyclonal anti-PKCβ1, and the polyclonal anti-PKCβ2 Abs were purchased from Santa Cruz Biotechnology. Mouse anti-SRp20 Ab was purchased from Zymed Laboratories. The polyclonal anti-JAK2, the polyclonal anti-phospho-Akt (Ser^{473}), the polyclonal anti phospho-Lyn (Tyr^{507}), and the polyclonal anti-Bcl-x_L Abs were purchased from Cell

*Division of Neurogenetics and Bioinformatics, Center for Neurological Diseases and Cancer, and †Department of Respiratory Medicine, Nagoya University Graduate School of Medicine, Nagoya, Japan; ‡Department of Medical Technology, Nagoya University School of Health Sciences, Nagoya, Japan; §Tokyo New Drug Research Laboratories II, Kowa Company, Tokyo, Japan; ¶Laboratory for Systems Biology and Medicine, Research Center for Advanced Science and Technology, University of Tokyo, Tokyo, Japan; and ‖Division of Biochemistry and Molecular Dentistry, Department of Developmental Medicine, Graduate School of Medical and Dental Sciences, Kagoshima University, Kagoshima, Japan

Received for publication May 2, 2006. Accepted for publication October 17, 2006.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported in part by grants from the Japanese Society for the Promotion of Science and the Japanese Allergy Foundation, the Kao Foundation for Arts and Sciences, and the ONO Medical Research Foundation.

[2] Address correspondence and reprint requests to Dr. Akio Masuda, Division of Neurogenetics and Bioinformatics, Center for Neural Disease and Cancer, Nagoya University Graduate School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya 466-8550, Japan. E-mail address: amasuda@med.nagoya-u.ac.jp

[3] Abbreviations used in this paper: PKC, protein kinase C; SCF, stem cell factor; DNP, dinitrophenol; HSA, human serum albumin; BMMC, bone marrow-derived mast cell; DN, dominant negative; siRNA, small-interfering RNA; Dox, doxycycline; ChIP, chromatin immunoprecipitation; PCA, passive cutaneous anaphylaxis; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium.

Signaling Technology. PE-conjugated anti-IgE Ab was purchased from eBioscience.

Cells

The RBL2H3 rat mast cell line was obtained from the Cell Resource Center for Biomedical Research (Tohoku University, Sendai, Japan) and was grown in RPMI 1640 with 10% FCS.

Bone marrow-derived mast cells (BMMCs) were derived from femoral bone marrow cells of BALB/c mice. Cells were cultured with IL-3 (10 ng/ml) for 3 wk and then cultured with IL-3 plus SCF (10 ng/ml each) for 1 wk. The cells consisted of >98% mast cells assessed by toluidine blue staining and FACS analysis of cell surface expression of c-*kit* and FcεRI.

For PMA/ionomycin stimulation, cells were washed twice and incubated (2×10^6 cells/ml) in fresh culture medium for 6 h, then stimulated with PMA (10 ng/ml) plus ionomycin (1 μg/ml). For the cross-linking of FcεRI on mast cells, cells were sensitized by incubating for 2 h with 1 μg/ml anti-DNP IgE in culture medium, washed, incubated (2×10^6 cells/ml) for 6 h in culture medium and stimulated with 50 ng/ml DNP-HSA.

Immunohistochemical analysis

BALB/c mice were sacrificed by terminal anesthesia. Tissues were fixed in 4% paraformaldehyde overnight, embedded in paraffin, and 3-μm-thick sections were cut from the paraffin blocks. Double stainings of immunohistochemistry and Alcian blue were performed as follows: sections were deparaffinized in xylene and hydrated with graded alcohols. Ag retrieval was performed in 10 mM citrate buffer (pH 6.0) using a microwave oven for 15 min. After blocking of endogenous peroxidase in H_2O_2(0.3%)-added methanol for 20 min and incubating with normal goat serum for 20 min, the primary Abs to GATA-1 (dilution 1/200) and GATA-2 (dilution 1/800) were applied and incubated overnight at 4°C. Staining was performed using Histofine Simplestain MAX-PO kit (Nichirei Biosciences). After washing in PBS, the sections were incubated with secondary Ab for 30 min at room temperature, followed by washing in PBS. The Ag-Ab immunoreaction was visualized using diaminobenzidine as a chromogen. Negative controls were processed by omitting the primary Ab-incubating step. After immunohistochemical staining, sections were rinsed in 1% Alcian blue solution for 30 min, followed by counterstaining with Kernechtrot, dehydration, and mounting.

Mammalian expression plasmids

To express reverse tet-responsive transcriptional activator in mast cells, the CMV promoter of the pTet-ON plasmid (BD Clontech) was substituted by the *EF1α* promoter (pEF1α-Tet-ON). The *EF1α* promoter was isolated from pEFBOS-Flag vector with *Hin*dIII-*Eco*RI and cloned into the *Hin*dIII-*Eco*RI site of the pEGFP-N1 vector. Then, this vector was partially digested with the *Xho*I-*Eco*RI site, and a 1.5-kbp product containing the EF1α promoter was cloned into the *Xho*I-*Eco*RI sites of pTet-ON (partially digested; 6.7-kbp fragment).

To generate the inducible expression plasmid for the dominant-negative (DN)-GATA (pTRE2hyg-DN-GATA), the cDNA encoded DN-GATA was released from ΔNn plus C plus NF plasmid (14) by *Nhe*I and subcloned into the *Nhe*I site of pTRE2hyg (BD Clontech). ΔN plus C plus NF consists of the Flag-tagged N-terminal zinc finger of mouse GATA-1.

pGATA-1&2-Luc, which is designed to measure transcriptional activity of both GATA-1 and GATA-2, were purchased from Panomics. pNFκB-Luc was described previously (16).

Generation of stable transfectants

RBL2H3 cells were transfected with 5 μg of pEF1α-Tet-ON vector using a T820 electroporation system (BTX). Transfectants were selected with G418 (0.5 mg/ml). After 4 wk, resistant clones were screened by transient transfections with the pTRE-Luc reporter plasmid and a luciferase assay for clones with low background expression and high Dox-dependent inducing of the rtTA regulatory protein. Selected stably transfected pEF1α-Tet-ON RBL2H3 cell clones served as non-DN-GATA-inducible control cells in all experiments (described as a parental clone). In a second selection step, the pTRE2hyg-DN-GATA plasmid was introduced into these clones to allow selection of stably transformed cells in the presence of hygromycin (1.2 mg/ml). Several G418- and hygromycin-resistant cell clones were isolated by single-cell cloning and screened by immunoblot analysis for clones with low background expression and high Dox-dependent inducing of DN-GATA protein. The induction of DN-GATA expression was performed by addition of Dox (1 μg/ml) into culture medium in all experiments. In addition, the parental clone was transfected with pTRE2hyg plasmid alone and selected with hygromycin as described above. Stably transfected pTRE2hyg cell clones served as non-DN-GATA-inducible control cells (described as a TRE clone).

Luciferase assay

DN-GATA-expressing or parental clones were transiently transfected with 3.5 μg of pGATA-1&2-Luc plasmid and 0.1 μg of pRL/SV40 (an internal control) by electroporation as described above.

BMMCs were transiently transfected with 3.5 μg of pGATA-1&2-Luc plasmid and 0.1 μg of pRL/SV40 (an internal control) using HVJ vector (Genome-One-Neo purchased from Ishihara Sangyo) according to the manufacturer's instruction.

Forty-eight hours after the transfection, the luciferase activity was measured by using the Dual-Luciferase Reporter Assay System (Toyo Ink) according to the manufacturer's instructions.

Transfection of small-interfering RNA (siRNA)

The siRNA used for transfections were: GATA-1 (D-045656-04; Dharmacon), GATA-2 (D-062114-02; Dharmacon), and control (nontargeting siRNA, D-1210-02; Dharmacon). The total amount of 200 pM siRNA duplexes was transfected into BMMCs (0.5×10^6 cells) by using 1 arbitrary unit of HVJ-E vector (Genome-One-Neo) according to the manufacturer's instruction.

Passive cutaneous anaphylaxis (PCA) reaction

Eleven-week-old BALB/c mice (SLC) were purchased (Japan SLC). Anti-DNP IgE (2.5 mg/ml) and experimental molecules in 20 μl of PBS were applied to the ear of the mice by intradermal injection. A total of 0.5 μl of 1 mM K-7174 or DMSO (control) was injected into the ears of mice intradermally simultaneously with anti-DNP IgE injections. Sixteen hours later, 2% Evans blue (Wako) and Ag, 1 mg/ml DNP-HSA (Sigma-Aldrich), in 100 μl of PBS was injected via the tail vein (i.v.). Thirty minutes later, the mice were sacrificed by terminal anesthesia, and photographed. Both ears were removed, and the extravasated Evans blue was extracted by incubating the skin samples in 99% *N*, *N*-dimethylformamide for 24 h at 55°C. The supernatant was collected by centrifugation and OD was read at 620 nm. PCA response was quantified by dye extraction from IgE-injected and PBS-injected ears.

Microarray analysis

The parental (cont) and DN-GATA-expressing clones (01 and 02) were incubated with Dox for 2 days. Then, cells were stimulated with PMA/ionomycin for 4 h, and total cellular RNA was prepared using TRIzol reagent (Invitrogen Life Technologies) and purified with a GenElute mammalian total RNA elution kit (Sigma-Aldrich). Relative mRNA levels were assessed using the Affymetrix gene chip Rat Expression Array 230A, containing probes for ~16,000 genes. Samples were processed into cRNA, hybridized to chips, and scanned at the Takara bio Dragon Genomics Center (Mie, Japan). Data sets were analyzed using Microarray Suite software (version 5.0). For each gene, a Wilcoxon signed-rank test was applied to the absolute signal intensities in the DN-GATA vs the control data set. Transcripts were defined as up-regulated or down-regulated only when identified as significantly different ($p < 0.005$).

RNA and cDNA preparation

RNA was isolated by using the QuickGene-810 system (Fuji). During this purification process, the RNA was DNase treated. Reverse transcription reactions were performed with 2 μg of total RNA using Superscript II (Invitrogen Life Technologies) reverse transcriptase as previously described (17).

Real-time PCR analysis

The quantitative real-time PCRs were performed using SYBR Green real-time PCR master mix (Toyobo) in a MX3000P (Stratagene) according to manufacturer's protocols. Primer sequences were as follows: *PKCβ1* sense, 5′-GCTAGAGACAAGCGAGACA-3′; antisense, 5′-ACACAGGC TCAGCGATGGA-3′; *PKCβ2* sense, 5′- TGTCATTCAAGCTCAACAG CTATCA-3′; antisense, 5′- ACACAGGCTCAGCGATGGA-3′; *GAPDH* sense, 5′-CTTCATTGACCTCAACTACATG-3′; antisense, 5′-TGTCAT GGATGACCTTGGCCAG-3′.

Western blotting analysis

Nuclear and cytoplasmic lysate preparation, and Western blotting were performed as previously described (14, 18).

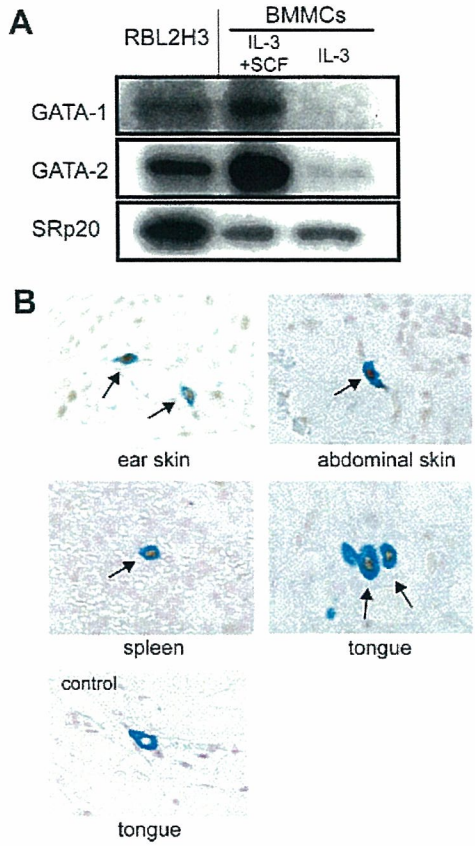

FIGURE 1. *A*, Expression of GATA transcriptional factor in RBL2H3 and BMMCs. BMMCs were cultured with IL-3 for 3 wk and then cultured with IL-3 plus SCF or IL-3 alone for 1 wk. Nuclear extracts were harvested and Western blot analyses were performed using GATA-1, GATA-2, or SRp20 (control) specific Ab. *B*, Expression of GATA-2 in tissue mast cells. A section was stained with Alcian blue and GATA-2-specific Ab. Arrows indicate mast cells that express GATA2 protein. The data of second Ab alone (control) is shown to rule out the possibility of nonspecific staining by anti-GATA-2 Ab.

β-Hexosaminidase release assay

β-Hexosaminidase release assay was performed as previously described (19). Results were expressed as percentage of total β-hexosaminidase activity present in the cells. Results were expressed as percentage of total β-hexosaminidase activity present in the cells.

Cytokine ELISA

Murine IL-4, IL-12p40, and TNF-α immunoassay kits (BD Pharmingen), murine IL-13 ELISA and rat IL-13 ELISA kits (R&D Systems), and rat IL-4, IL-12p40, and TNF-α immunoassay kits (BioSource International) were used according to the manufacturer's instructions.

Measurement of cell proliferation

Proliferation was measured by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium (MTS) assay as previously described (20). Corrected absorbance at 490 nm was calculated by subtracting the background absorbance (medium alone).

Detection of apoptosis

Apoptosis of mast cells was measured using the Annexin V^{FITC} apoptosis detention kit according to the manufacturer's instruction (MBL). The flow cytometric analysis was performed using a FACSCalibur flow cytometer (BD Biosciences).

Detection of FcεRI expression on mast cells

Cells were sensitized by incubating for 2 h with 1 μg/ml anti-DNP IgE in culture medium. Then cells were stained with PE-conjugated anti-IgE Ab.

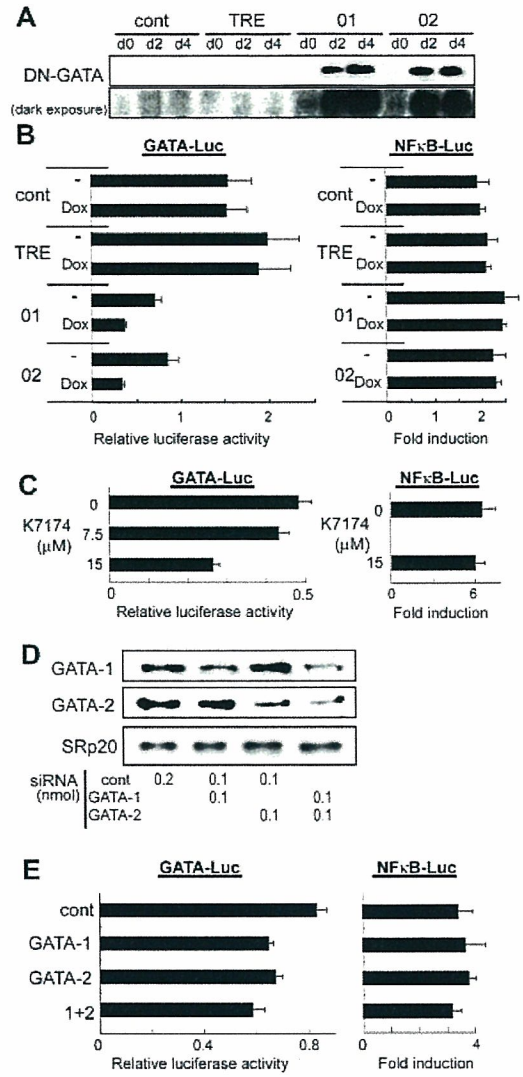

FIGURE 2. Suppression of GATA activity in mast cells. *A*, Inducible expression of DN-GATA in RBL2H3 cells. Western blot analysis was performed with nuclear extracts from the parental (cont), the clone transfected with vector alone (TRE), and DN-GATA-expressing clones (01 and 02) treated with Dox for the indicated days. The membrane was probed with an anti-Flag Ab, as DN-GATA is tagged with Flag. *B*, Repression of GATA transcriptional activity by DN-GATA. Cells were transiently transfected with 1 μg of pGATA-1&2-Luc plasmid and 0.1 μg of pRL/SV40. Cells were incubated with Dox or not for 2 days and then luciferase activities were measured. *C*, Repression of GATA transcriptional activity by K-7174. BMMCs were transiently transfected with 1 μg of pGATA-1&2-Luc plasmid and 0.1 μg of pRL/SV40 using HVJ vector. After 32 h, K-7174 was added to culture medium and incubated for 16 h. Then luciferase activities were measured. *D*, Inhibition of GATA protein production by siRNA. BMMCs were transfected with the indicated amount of nonspecific control siRNA (cont) or specific siRNA for GATA-1 or GATA-2 using HVJ vector. After 2 days, nuclear extracts were harvested and Western blot analyses were performed using GATA-1, GATA-2, or SRp20 (control) specific Ab. *E*, Repression of GATA transcriptional activity by siRNA. BMMCs were transfected with indicated amounts of nonspecific control siRNA (cont) or specific siRNA for GATA-1 or GATA-2 using HVJ vector. After 2 days, luciferase activities were measured. To examine NF-κB activity, cells were transfected with 1 μg of pNFκB-Luc and 0.1 μg of pRL/SV40. Cells were stimulated with IgE cross-linking for 16 h or not. Units of luciferase activity were normalized based on values of pRL/SV40 activity for transfection activity (relative luciferase activity). The fold inductions were calculated as follows: (relative luciferase activity of stimulated cells)/(relative luciferase activity of unstimulated cells). A typical result of at least three independent experiments is shown.

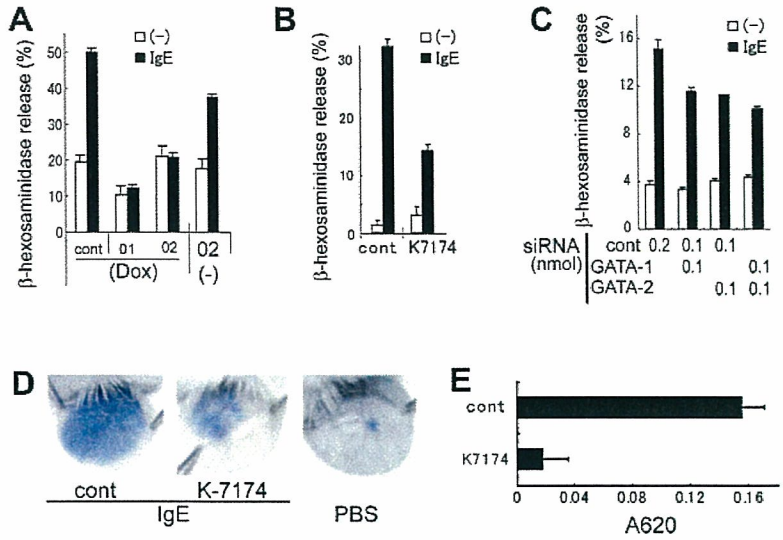

FIGURE 3. Inhibition of degranulation by suppression of GATA activity. *A–C*, Degranulation was assessed by measuring the release of β-hexosaminidase in the supernatant. *A*, The parental (cont) and DN-GATA-expressing clones (01 and 02) were incubated with Dox or not (−) for 2 days and then stimulated with IgE cross-linking. *B*, BMMCs were incubated with K-7174 (15 μM) or DMSO (cont) for 16 h and then stimulated with IgE cross-linking. *C*, BMMCs were transfected with indicated amount of nonspecific control siRNA (cont) or specific siRNA for GATA-1 or GATA-2 using HVJ vector. After 2 days, cells were stimulated with IgE cross-linking. *D*, Typical photographs of the PCA reaction from among five tests. *E*, Analytical data regarding the extravasated Evans blue. A typical result of three independent experiments is shown. Data are expressed as A620 = absorbance 620 nm of IgE-sensitized ear minus absorbance 620 nm of saline-injected ear. The error bars represent SD values. The experiments were done in triplicate. The error bars represent SD values.

After washing, the cells were resuspended and analyzed using a FACS-Calibur flow cytometer (BD Biosciences).

Detection of JNK phosphorylation

Human phospho-JNK (pan) ELISA kit (R&D Systems) was used according to the manufacturer's instruction. This assay also recognizes mouse and rat phospho-JNK.

Chromatin immunoprecipitation (ChIP) assay

ChIP assay was performed according to the manufacturer's instruction (Upstate Biotechnology). The quantitative real-time PCRs were performed using Real-time PCR master mix (Toyobo) in a MX3000P (Stratagene) according to the manufacturer's protocols. Primer sequences were as follows: the *PKCβ* promoter-specific primers are as follows: PKCβ pro1 (−927 to −784): sense, GGTACTTACAACCACATAGACA; antisense, CT TGCTCCAACGAACCCTTAGA; probe, FAM-CTCTGCTTGCTCCAACG AACCCTT-TAMRA. PKCβ pro2 (−261 to −187): sense, CCTTGAACCC TTCGGGTACT; antisense, CCAGCCAAGTGTTCTTAGCC; probe, FAM-CCGCCCAGAGCCGCCAGCT-TAMRA. Intron2 of *JAK2*-specific primers were as follows: sense, TGTATGGGAAGGGTTTGACTCC; antisense, GG CAAAGGACAAGTCTGTGC; probe, FAM-CACAAGAGGGCAGCACCA CCAGGC-TAMRA.

Results

Expression of GATA transcriptional factors in differentiated mast cells

To investigate the role of GATA transcriptional factors in mast cells, we examined a well-established basophilic mast cell line RBL2H3 and BMMCs. As shown in Fig. 1*A*, both GATA-1 and GATA-2 were expressed in the RBL2H3 cells, as previously described (Fig. 1*A*) (10). In BMMCs prepared with IL-3 alone, GATA-2 expression was observed, although GATA-1 expression was below the level of detection. When the culture medium was supplemented with the SCF, BMMCs expressed detectable amounts of the GATA-1 protein and significant amounts of the GATA-2 protein (Fig. 1*A*). Because SCF is known to induce maturation of mast cells (21), these results indicate that GATA-1 and GATA-2 are abundantly expressed in differentiated BMMCs.

Although various mast cell lines and BMMCs have been reported to express GATA-1 or GATA-2 (10), the in vivo expression of GATA proteins in mast cells remains controversial (8, 9, 22). To confirm the expression of GATA proteins in mast cells distributed in tissue, immunohistochemical analysis was performed using GATA-specific Abs along with Alcian blue staining. As shown in Fig. 1*B*, GATA-2 expression was observed in mast cells in various mouse tissue, such as the skin on the ear and the abdomen, the tongue, and the spleen (Fig. 1*B*). In contrast, GATA-1 expression was below the level of detection in tissue mast cells, although the nuclei of megakaryocytes were clearly stained with the same Ab (data not shown). These data suggest that GATA-2 protein is expressed in mast cells in vivo as well as in mast cell lines and BMMCs.

Suppression of GATA activity in mast cells

In an attempt to specifically repress the activities of GATA-1 and GATA-2, we used the inducible overexpression of a DN form of GATA (DN-GATA), the specific GATA inhibitor (K-7174) and siRNA technology.

DN-GATA is a deletion mutant of GATA-1, which consists of the N-terminal zinc finger of GATA-1. We previously described that the overexpression of this deletion mutant significantly inhibited the transactivation of *IL-13* promoter in mast cells (14). Because we could not obtain cell lines that express DN-GATA stably, we selected the tetracycline-inducible expression system for the expression of DN-GATA in the RBL-2H3 cells. Two clones expressing this deletion mutant were isolated for analyses. As shown in Fig. 2*A*, DN-GATA expression was significantly induced in these cell lines in the presence of Dox. To confirm whether this deletion mutant actually worked in a DN fashion, we examined GATA transcriptional activity using a luciferase reporter plasmid designed to measure transcriptional activity of both GATA-1 and GATA-2 (pGATA-1&2-Luc). As shown in Fig. 2*B*, the absence of Dox resulted in a moderate reduction in the GATA activity in these cell lines, suggesting that the background expression of DN-GATA can suppress GATA activity. However, a severe reduction was observed after Dox supplementation. In contrast, NF-κB activity did not affected by expression of DN-GATA (Fig. 2*B*). These results indicate that DN-GATA really worked in a DN fashion.

In BMMCs, we used K-7174 and siRNA to repress GATA activity. K-7174 was developed as a low m.w. anti-inflammatory drug; it is known to be a specific inhibitor of GATA (23). To examine whether K-7174 represses GATA activity in mast cells, BMMCs were transfected with pGATA-1&2-Luc using the HVJ vector, and GATA transcriptional activity was measured. As shown in Fig. 2*C*, K-7174 inhibited GATA transcriptional activity in BMMCs.

Transfection of BMMCs with GATA-1 and GATA-2 siRNAs by using the HVJ vector resulted in markedly decreased levels of

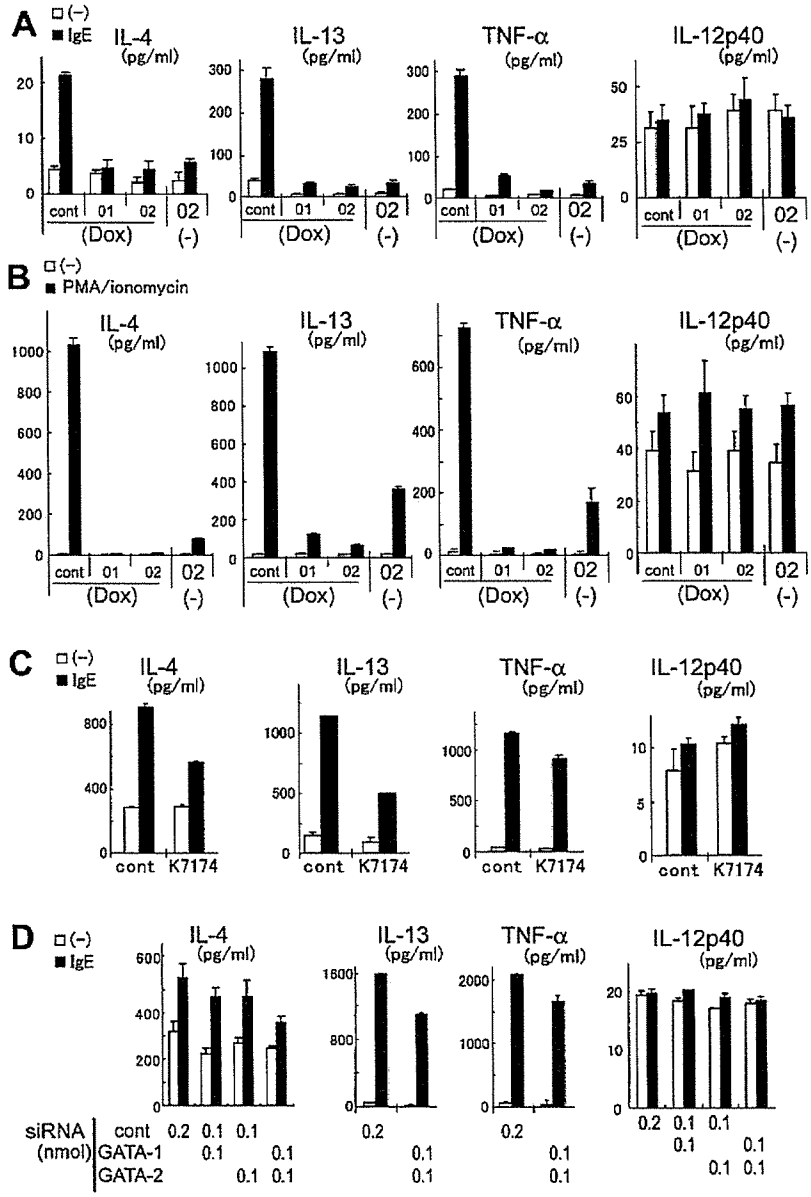

FIGURE 4. Inhibition of cytokine production by suppression of GATA activity. Cells were stimulated with IgE cross-linking or PMA/ionomycin. After 16 h, the cell-free culture supernatants were collected and cytokine ELISAs were performed. *A*, IgE cross-linking induced cytokine production from DN-GATA-expressing cells. The parental (cont) and DN-GATA-expressing clones (01 and 02) were incubated with Dox or not (−) for 2 days and then stimulated with IgE cross-linking. *B*, PMA/ionomycin induced cytokine production from DN-GATA-expressing cells. Cells were incubated with Dox or not for 2 days and then stimulated with PMA/ionomycin. *C*, IgE cross-linking induced cytokine production from BMMCs treated with K-7174 (15 μM) or DMSO (cont). Cells were incubated with K-7174 for 16 h and then stimulated with IgE cross-linking. *D*, IgE cross-linking induced cytokine production from BMMCs treated with siRNA. Cells were transfected with indicated amount of nonspecific control siRNA (cont) or specific siRNA for GATA-1 or GATA-2 using HVJ vector. After 2 days, cells were stimulated with IgE cross-linking. We confirmed that 16 h incubation did not significantly affect the number of cells (data not shown). The experiments were done in triplicate. The error bars represent SD values.

the GATA-1 and GATA-2 proteins, respectively, when compared with those obtained with the control siRNA (Fig. 2*D*). RT-PCR analysis showed that these siRNAs produced an ~70% reduction in both GATA-1 and GATA-2 mRNAs (data not shown). As shown in Fig. 2*E*, these siRNAs inhibited the GATA transcriptional activity in BMMCs. (Fig. 2*E*).

Because these three strategies (DN-GATA, siRNA, and K-7174) can repress the GATA activity within 2 days, the role of GATA in mast cells can be analyzed with minimum influences from the cell differentiation status.

Inhibition of degranulation by suppression of GATA activity

Degranulation is considered to be a major function of mast cells. To determine whether the GATA activity was required for degranulation, we investigated the granule release from the mast cells by measuring the extracellular activity of β-hexosaminidase, a marker enzyme for histamine-containing granules. As shown in Fig. 3*A*, DN-GATA-expressing cell lines that were not induced with Dox revealed a moderate inhibition of degranulation; severe suppression of GATA activity by Dox supplementation resulted in the complete inhibition of degranulation.

Furthermore, the suppression of degranulation was also confirmed in BMMCs in which the GATA activity was suppressed with GATA inhibitor or siRNA (Fig. 3, *B* and *C*). Both GATA-1 and GATA-2 siRNAs inhibited the degranulation from mast cells and the combination of these siRNA inhibited more significantly (Fig. 3*C*).

We next assessed the efficacy of the GATA inhibitor in suppressing degranulation from normal tissue-resident mast cells in vivo. The immediate hypersensitivity reaction was analyzed by local PCA. K-7174 or DMSO (control) was injected intradermally into the ears of mice simultaneously with anti-DNP IgE injections. On the next day, DNP-HSA together with Evan's blue was injected i.v. Within 5 min after this antigenic challenge, the mast cell-dependent PCA increased vascular permeability, causing local dye extravasation in the IgE-injected skin but not in the PBS-injected skin. As shown in Fig. 3, *D* and *E*, the size and color intensity of the

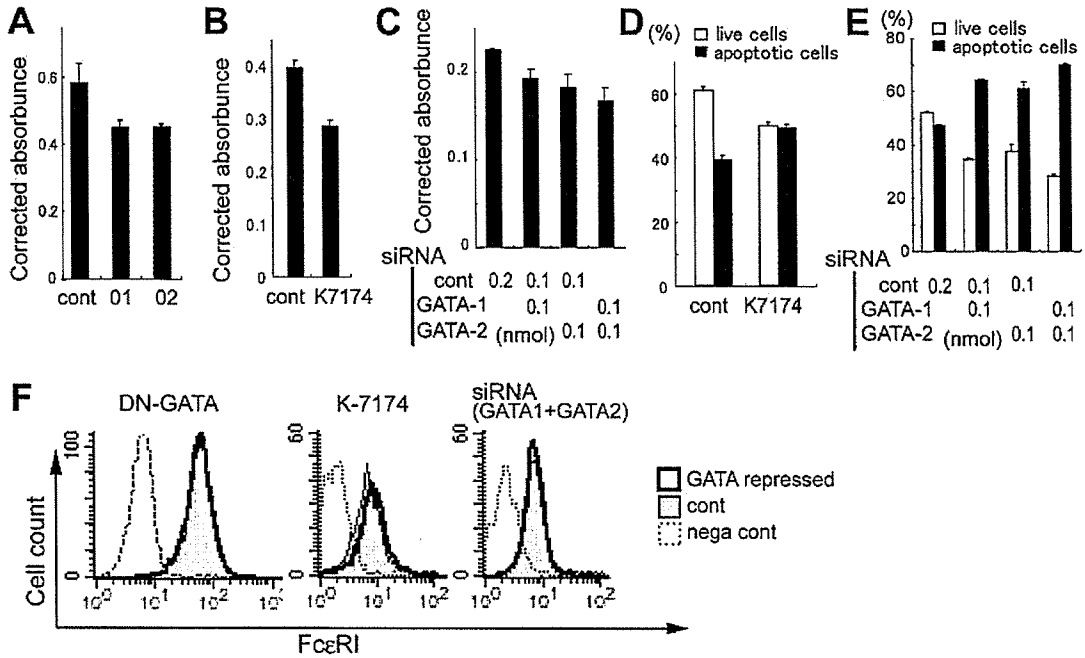

FIGURE 5. *A–C*, Inhibition of mast cell proliferation by repression of GATA activity. Cells (2×10^4 cells) were incubated in 96-well microplates. After 48 h, cell number was measured by MTS assay. Corrected absorbance was calculated as described in *Materials and Methods*. *A*, The parental (cont) and DN-GATA-expressing clones (01 and 02) were incubated with Dox for 2 days. Then cells were washed twice and incubated with Dox again. *B*, BMMCs (2×10^4 cells) were incubated with K-7174 (15 μM) or DMSO (cont) in RPMI 1640 plus 10% FCS supplemented with IL-3 plus SCF. *C*, BMMCs were transfected with nonspecific control siRNA (cont) or specific siRNA for GATA-1 or GATA-2 using HVJ vector. After 2 days, cells were washed twice and incubated in RPMI 1640 plus 10% FCS supplemented with IL-3 plus SCF. *D*, Promotion of mast cell apoptosis by repression of GATA activity. BMMCs were incubated with K-7174 (15 μM) or DMSO (cont) in RPMI 1640 plus 10% FCS for 14 h. Then, the cells were stained with Annexin V^{FITC} and propidium iodide and analyzed by flow cytometry. *E*, Promotion of mast cell apoptosis by repression of GATA activity. BMMCs were transfected with nonspecific control siRNA (cont) or specific siRNA for GATA-1 or GATA-2 using HVJ vector. After 2 days, cells were washed twice and incubated in RPMI 1640 plus 10% FCS for 16 h. Then, the cells were stained with Annexin V^{FITC} and propidium iodide, and analyzed by flow cytometry. *F*, Expression of FcεRI on mast cells. Cells were prepared as described for *A–C* and incubated with IgE for 2 h. Then cells were stained with anti-mouse IgE, or isotype match Ab (nega cont). After washing, the cells were resuspended and analyzed by flow cytometry. A typical result of at least three independent experiments is shown. The error bars represent SD values.

reaction at the sites of K-7174 injection were significantly decreased when compared with those at the sites injected with DMSO. These data clarify the importance of GATA transcriptional factors in degranulation from mast cells.

Inhibition of cytokine production by suppression of GATA activity

Because it has been reported that the GATA-binding sites in *IL-4* and *IL-13* promoters are necessary for their transcriptional activity in mast cells (13, 14), we examined whether the suppression of GATA activity would affect the cytokine production from mast cells. As shown in Fig. 4*A*, after IgE cross-linking, the production of IL-4, IL-13, and TNF-α was significantly inhibited in DN-GATA-expressing cell lines. In contrast, the production of IL-12p40 was not affected in these cells. A similar inhibition of cytokine production was also observed in cells stimulated with PMA and ionomycin (Fig. 4*B*). Although the cells induced with Dox showed more severe reductions in PMA/ionomycin stimulation, DN-GATA-expressing cell lines that were not induced with Dox also showed significant reduction of cytokine production, suggesting that mild suppression of GATA activity is enough for inhibition of the cytokine productions. Additionally, inhibition of cytokine production was also confirmed in BMMCs in which GATA activity was suppressed using a GATA inhibitor or siRNAs (Fig. 4, *C* and *D*).

Using the MTS assay, we next examined whether the repression of GATA activity inhibited proliferation of mast cells. As shown in Fig. 5, *A–E*, the suppression of GATA activity inhibited their proliferation and promoted apoptosis of mast cells.

Although it has been reported that GATA-1 is required for FcεRI expression on mast cells during their development (24), no change in FcεRI expression was observed by the suppression of GATA activity in our experiments (Fig. 5*F*). Thus, the role of GATA in developed mast cells appeared to be different from that in precursors and alteration of FcεRI expression is not the cause of the inhibited cytokine production and degranulation induced by the suppression of GATA activity in developed mast cells. Taken together, these data strikingly indicate the requirement of GATA activity for homeostasis and activation of mature mast cells.

GeneChip analysis of differential gene expression induced by GATA activity suppression

To identify GATA target genes that contribute to the biological effects of GATA on mast cells, we compared the transcripts of DN-GATA-expressing and nonexpressing cell lines stimulated with PMA and ionomycin by using an Affymetrix GeneChip. Approximately 15,000 of nearly 16,000 genes did not show a significantly different hybridization signal between DN-GATA-expressing cells and control cells. The results of important genes are shown in Table I. The transcription of several genes, which has been reported to be transactivated by GATA, was found to be decreased in DN-GATA-expressing cells; including *carboxypeptidase A*, *IL-4*, and *IL-13* (10, 13, 14). The decrease in *IL-4* and *IL-13* transcripts is consistent with the above-described ELISA

Table I. *Change in transcript expression in DN-GATA-expressing cells*[a]

Function	Gene Name	Fold Change	Genes 0.1-fold or Less Down-Regulated by DN-GATA Expression	
			Gene Name	Fold Change
Cytokine	*IL-3*	0.31	*Carboxypeptidase A I*	0.009
	IL-4	0.38	*MCP-1*	0.012
			Lymphocyte Ag 68	0.012
	IL-13	0.09	*Mast cell protease 8*	0.014
Cytokine receptor	*IL-1R type2*	0.33	*CXCR4*	0.019
	IL-2Rα	0.04	*PKC-binding protein ζ1*	0.021
	IL-4R	0.41	*IL-10Rα*	0.041
	IL-9R	2.14	*IL-2Rα*	0.044
			Cyclooxygenase 2	0.054
	IL-10Rα	0.04	*LCR1*	0.054
Chemokine	*MCP-1*	0.01	*Mcpt2*	0.058
			Tgfb1i4	0.067
	RTCK1	0.09	*Pacsin1*	0.088
Chemokine receptor	*CXCR4*	0.02	*IL-13*	0.095
	LCR1	0.05	*RTCK1*	0.095
Signal transduction	*Btk*	3.43		
	PLD2	0.22		
	PLCγ1	2.14		
	PKCβ	0.38		
Apoptosis	*Bcl-x_L*	0.47		
	Bcl-2A1	0.27		

[a] The parental (cont) and DN-GATA-expressing clones (02) were incubated with Dox for 2 days. Then, cells were stimulated with PMA/ionomycin for 4 h. Total RNA was extracted and analyzed for transcript change by Affymetrix GeneChip. Values are fold change relative to signal intensities in the DN-GATA to those in control.

data. Interestingly, the levels of the *Bcl-x_L* and *Bcl-A1* genes, which prevent apoptosis of mast cells (20, 25), were decreased in DN-GATA-expressing cells, indicating the contribution of these genes to GATA-dependent cell survival. Among the various transcripts that encode proteins related to signal transduction, the level of the *PKCβ* gene was decreased. Although the levels of the Bruton tyrosine kinase (*Btk*) gene and the phospholipase C (*PLC*)-*γ1* gene were increased, these genes have been known to positively regulate downstream signals and mast cell activation (26, 27). Therefore, these up-regulations are not the cause of mast cell inactivation occurring due to the repression of GATA activity. Probably, these up-regulations are a secondary effect of the repression of GATA activity. Real-time PCR and Western blot analyses showed that *PKCβI*, *PKCβII*, and *Bcl-x_L* were significantly down-regulated in BMMCs treated with siRNA or the GATA inhibitor as well as in DN-GATA-expressing cell lines (Fig. 6, *A* and *B*).

Signal transduction from FcεRI in GATA-repressed mast cells

We then examined the downstream signals of PKCβ in GATA-repressed mast cells after FcεRI cross-linking. Regarding the signal transduction pathway from FcεRI, it has been reported that PKCβ is involved in JNK and Akt activation (27, 28). Surprisingly, we found that the phosphorylation of both JNK and Akt (Ser^{473}) was significantly inhibited in DN-GATA-expressing cells (Fig. 6*C*). In contrast, Lyn, which is rapidly phosphorylated after FcεRI cross-linking and upstream of PKCβ (27, 29), was normally phosphorylated in DN-GATA-expressing cells (Fig. 6*D*). These results indicate the inactivation of downstream signal transduction of PKCβ in DN-GATA-expressing cell lines, presumably due to the decreased PKCβ expression.

Histone acetylation status after suppression of GATA activity in mast cells

It has been reported that GATA transcriptional factors regulate tissue-specific gene expression through the modification of histone acetylation (30). To investigate the possibility of histone modification by GATA in the regulation of the *PKCβ* gene in mast cells, we examined H3 and H4 acetylation in DN-GATA-expressing cell lines after the suppression of GATA activity.

We applied the ChIP assay using specific primers for the *PKCβ* promoter. As shown in Fig. 6*E*, H3 and H4 acetylation in the *PKCβ* promoter region was significantly decreased in DN-GATA-expressing cells, although acetylation in the control gene (*JAK2*) did not change. Additionally, the down-regulation of histone acetylation at the *IL-4* promoter and *Bcl-x* promoter regions was observed in GATA-repressed cells (data not shown). A search in the nucleotide sequence of the PKCβ promoter for the potential binding sites of transcriptional factors was conducted using TRANSFAC (www.motif.genome.ad.jp; cutoff score = 85) and the GATA-binding site was found in the region between −905 and −893. The ChIP assay indicated that binding of GATA to this region was decreased in DN-GATA-expressing cells (Fig. 6*E*, PKCβpro1). These results indicate that GATA regulates *PKCβ* gene expression through histone modification.

Discussion

In this study, we have shown the existence of GATA proteins in mast cell lines, BMMCs, and tissue mast cells and that the repression of GATA activity significantly inhibits cytokine production, degranulation, and survival of mast cells, indicating that the GATA activity is indispensable for homeostasis and activation of mature mast cells. Microarray analysis has revealed that the transcription of several cytokines, their receptors, and apoptosis-related genes was under the control of GATA transcriptional factors in mast cells. Among the various genes related to signal transduction, *PKCβ* was down-regulated in GATA-repressed mast cells. Lyn, which is rapidly phosphorylated after FcεRI cross-linking in the upstream of PKCβ, was normally phosphorylated in the GATA-repressed mast cells. In contrast, downstream signals such as the phosphorylation of JNK and Akt were abrogated. Finally, we have shown that histone acetylation at the *PKCβ* gene region

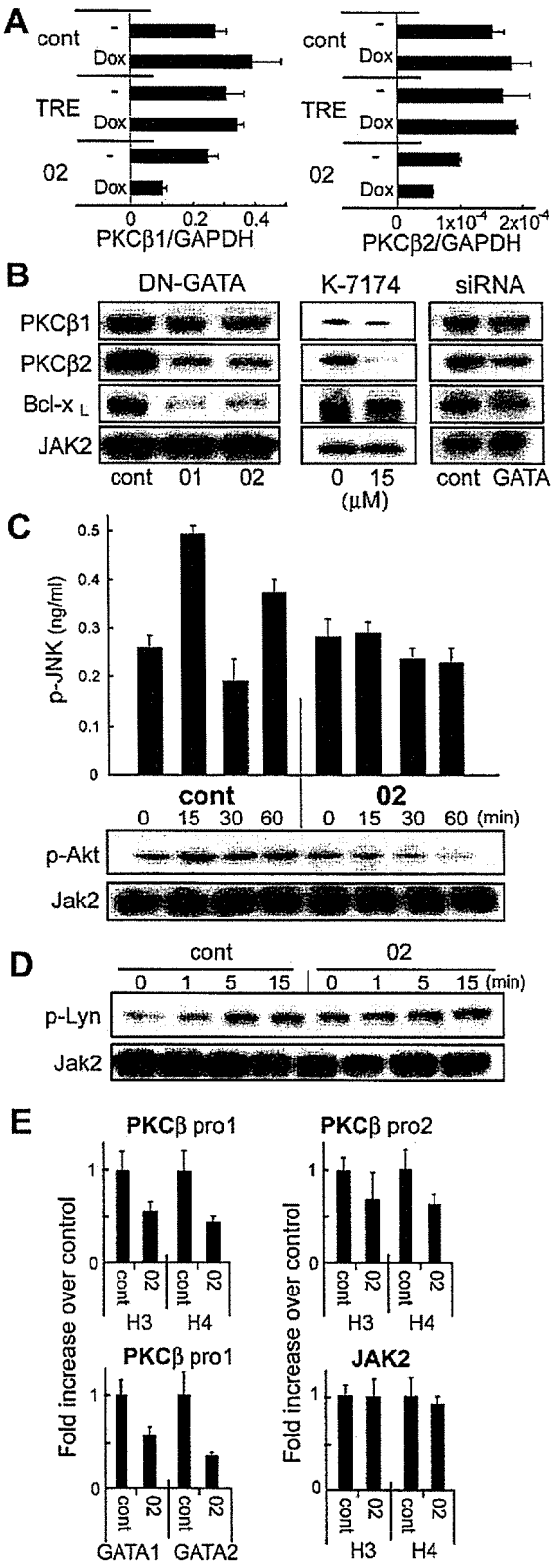

FIGURE 6. *A*, Real-time PCR analysis of PKCβ mRNA in DN-GATA-expressing cells. The parental (cont), the clone transfected with vector alone (TRE), and DN-GATA-expressing clones (02) were incubated with Dox or not (−) for 2 days. Total RNA was reverse-transcribed, and *PKCβ1*, *PKCβ2*, and *GAPDH* cDNAs were amplified by real-time PCR. The error bars represent SD values. *B*, Western blot analysis of PKCβ1, PKCβ2, and Bcl-x_L expression. DN-GATA-expressing clones were incubated with Dox for 2 days (DN-GATA). BMMCs were incubated with K-7174 (15 μM) or not for 16 h (K-7174). BMMCs were transfected with nonspecific control siRNA (cont) or specific siRNAs for GATA-1 and GATA-2 (GATA), and incubated for 48 h. *C*, Inhibition of signal transduction by repression of GATA activity. The parental (cont) and DN-GATA-expressing clones (02) were incubated with Dox for 2 days. Cells were washed and resuspended in RPMI 1640 plus 10% FCS. After 6 h, cells were stimulated with IgE cross-linking for the indicated time and cell lysates were harvested. Phosphorylation of JNK was analyzed using the phospho-JNK-specific ELISA kit. Phosphorylation of Akt was analyzed by Western blot. *D*, Phosphorylation of Lyn in DN-GATA-expressing cells. Cell lysates were harvested as described for Fig. 6*C*. Western blot analysis was performed using phospho-Lyn-specific Ab. *E*, Acetylation levels of histone H3 and H4 at the *PKCβ* gene. The parental (cont) and DN-GATA-expressing clones (02) were incubated with Dox for 2 days. For the analysis of histone acetylation, the ChIP assays were conducted using anti-acetyl H3 or H4 Ab. For the analysis of binding of GATA, anti-GATA-1 or GATA-2 Ab was used. ChIP assays were quantified by real-time PCR using probes specific to the *PKCβ* promoter (two regions were analyzed; pro1, −927 to −784 and pro2, −261 to −187) and JAK2 intron2 (JAK2). Results are expressed as the fold increase over the levels detected in the control cells after correcting for differences in the amount of starting (input) chromatin material. The error bars represent SD values.

was significantly down-regulated in the GATA-repressed mast cells.

Mast cell precursors leave bone marrow, migrate in the blood, and differentiate into mature cells after tissue invasion (31). We have shown that the GATA-2 protein is expressed in mast cells distributed in various tissues in mice (Fig. 1*B*). In addition, it has been reported that mouse peritoneal mast cells express the GATA-1 protein (9) and that human skin mast cells express the GATA-2 protein (8). Therefore, it appeared that differentiated mast cells express GATA-1 or GATA-2. Because previous reports have shown that the *GATA-2* mRNA is highly expressed in the undifferentiated mast cell lineage and down-regulated during its differentiation (22), the GATA-2 expression in mature mast cells may not be so abundant.

It has been indicated that GATA transactivates gene transcription through the regulation of chromatin accessibility. For example, GATA-3 interacts with methyl CpG-binding domain protein-2 that regulates DNA methylation in the inactivation of chromatin and promotes Th2 cytokine expression (32). GATA-1 associates with the CREB-binding proteins CBP/p300, coactivating factors that have intrinsic histone acetyl transferase activity, and promotes *β-globin* gene expression (30).

The *PKCβ* gene encodes two mRNAs, namely, *PKCβI* and *PKCβII*, which originate from the alternative splicing of the C-terminal exons (33). In the present study, we have shown that the repression of GATA activity significantly decreased both *PKCβI* and *PKCβII* expression (Fig. 6, *A* and *B*). Although the transcriptional initiation site has been reported (34), little is known about the transcriptional regulation of *PKCβ*. Because our data indicated that histone acetylation and binding of GATA protein in the *PKCβ* promoter was decreased after the repression of GATA activity (Fig. 6*E*), it is quite conceivable that chromatin accessibility contributes to the transcriptional regulation of *PKCβ*. Interestingly, our microarray data indicated that the suppression of GATA activity down-regulated phospholipase D2 (*PLD2*) expression (Table I), which is necessary for PKC activation in mast cells (35). *PKCβ* activity may be suppressed through this pathway in addition to its decreased expression. Actually, *PKCβ* activity was significantly suppressed after the repression of GATA activity, as our experiments on signal transduction indicated (Fig. 6, *C* and *D*).

Although mast cells express several isoforms of PKC (36), various reports have indicated the importance of PKCβ in the signal