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Methodology article

Multigene family isoform profiling from blood cell lineages

Grant Dewson[1], Edward C Conley[2] and Peter Bradding*[3]

Address: [1]Biochem. Toxicol., CMHT, Leicester University, Lancaster Road, Leicester, LE1 9HN, UK, [2]APBiotech Inc. R&D Unit, Forest Farm Laboratories, Whitchurch, Cardiff, CF14 7YT, UK and [3]Division of Respiratory Medicine, Institute for Lung Health, University of Leicester Medical School, Glenfield Hospital, Leicester, LE3 9QP, UK

E-mail: Grant Dewson - gd11@le.ac.uk; Edward C Conley - ed.conley@uk.amershambiosciences.com; Peter Bradding* - pbradding@hotmail.com

*Corresponding author

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Abstract

Background: Analysis of cell-selective gene expression for families of proteins of therapeutic interest is crucial when deducing the influence of genes upon complex traits and disease susceptibility. Presently, there is no convenient tool for examining isoform-selective expression for large gene families. A multigene isoform profiling strategy was developed and used to investigate the inwardly rectifying K^+ (Kir) channel family in human leukocytes. Comprised of seven subfamilies, Kir channels have important roles in setting the resting membrane potential in excitable and non-excitable cells.

Results: Gene sequence alignment allowed determination of "islands" of amino acid homology, and sub-family "centred" priming permitted simultaneous co-amplification of each family member. Validation and cross-priming analysis was performed against a panel of cognate Kir channel clones. Radiolabelling and diagnostic restriction digestion of pooled PCR products enabled determination of distinct Kir gene expression profiles in pure populations of human neutrophils, eosinophils and lung mast cells, with conservation of Kir2.0 isoforms amongst the leukocyte subsets. We also identified a Kir2.0 channel product, which may potentially represent a novel family member.

Conclusions: We have developed a novel, rapid and flexible strategy for the determination of gene family isoform composition in any cell type with the additional capacity to detect hitherto unidentified family members and verified its application in a study of Kir channel isoform expression in human leukocytes.

Background

Identification of gene family expression profiles in individual cells is an important means of describing signal transduction selectivity and specificity. Thus, as the human genome project reaches completion [1], much emphasis is being placed on determining how combinations of gene expression influence complex traits and disease susceptibility. For families of receptors, channels, enzymes or other proteins that are potential therapeutic targets, the ability to identify cell-specific isoform expression from the many potentially encoded by the genome is crucial. Since coding portions of the human genome will soon be entirely determined, simple typing procedures need to be developed that accommodate all isoforms within known gene families. Conventional approaches to isoform profiling are based on synthesis of multiple sets

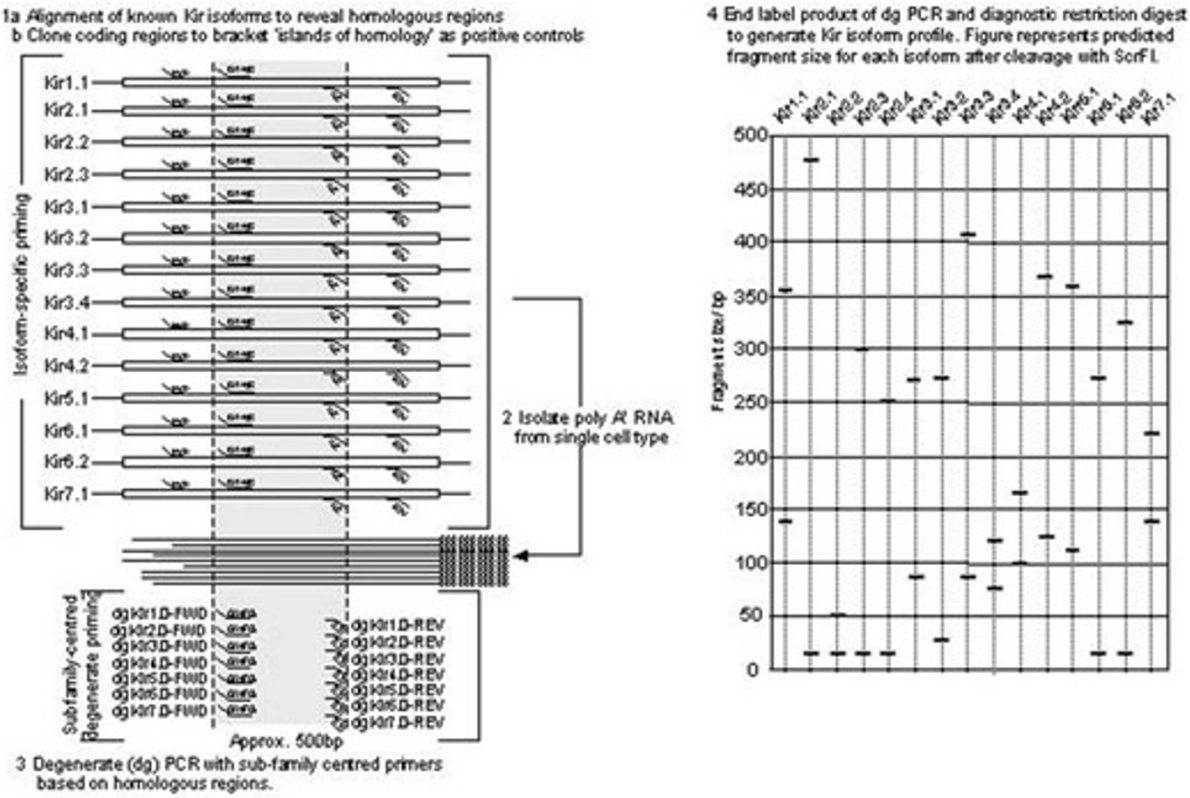

Figure 1
Kir family isoform profiling strategy. 1) Alignment of Kir channel isoform nucleic acid sequences revealed regions of high sequence similarity. Two conserved motifs, G(Y/F)G and ILE, in the pore and C terminal domains respectively, were selected and Kir isoforms cloned. 2) Poly A^+ RNA was isolated from a single cell type and 3) degenerate (*dg*) PCR performed with sub-family centred primers. Degenerate PCR products were radio end-labelled, recovered free of unincorporated nucleotides and cleaved with frequent-cutting restriction enzyme (ScrF I) and resolved by agarose gel electrophoresis.

of "gene-specific" oligonucleotides priming multiple reverse transcriptase-polymerase chain reactions (RT-PCR). However, because of the small amounts of mRNA sampled from many clinically-derived tissue samples, multiple independent RT-PCR reactions using "gene-specific" primer sets are difficult to standardise across large gene families. In particular, gene-specific RT-PCRs usually employ heterogeneous conditions to identify single bands with predicted mobilities on gels, and "absolute" typing is not feasible without sequence determination, which is costly and time-consuming. We faced these problems in studies which required isoform profiling from small numbers of blood-derived leukocytes, and in response, aimed to develop a flexible approach capable of automated "readout" of gene isoform composition per single cell type. The resulting strategy can be applied when all isoforms of a given gene subfamily are known, but also has the potential to identify novel unidentified family members. The method can thus be used to determine isoform selectivity within signalling complexes in single cell types.

As a model we have investigated the inwardly rectifying K^+ (Kir) channel gene family in human leukocytes. The Kir family is comprised of seven subfamilies (Kir 1.x to 7.x) based on degree of similarity of their primary amino acid sequences [2]. Each subfamily has distinct electrophysiological properties in terms of current rectification and channel gating. In spite of the apparently widespread expression of many of these K^+ channel family members in many tissues, their role in mammalian physiology is uncertain. When present in cell plasma membranes, they often contribute to or dominate the resting membrane

potential. In excitable cells such as neurons or smooth muscle this will have important effects in setting the threshold for cell activation. However, inwardly-rectifying K^+ currents have also been described in many non-excitable cells, including epithelium, endothelium and granulocytes [3–5], where the setting of resting membrane potential may also influence stimulus-secretion coupling. Their expression therefore in human leukocytes may suggest important immunomodulatory roles. Both human eosinophils and rodent mast cells express membrane currents at rest consistent with the presence of Kir 2 family members, and express mRNA for Kir 2.1 [5,6]. Neutrophils also have a negative resting membrane potential in association with an inwardly-rectifying K^+ current, which is likely to be carried by a Kir family member although this has not been identified [7]. However, there are several examples where two or more Kir family members are expressed in the same cell, for example, rat ventricular myocardium which expresses Kir 2.1, probably expresses other Kir 2, 3, and 6 family members [8]. In this paper we report the use of a novel isoform gene profiling strategy in identifying Kir gene expression in human neutrophils, eosinophils and lung mast cells (HLMC), all of which are important for innate immunity, and which are implicated in the pathogenesis of many diseases.

Results

Isoform profiling strategy

In our strategy for isoform profiling applied to the human Kir gene family, sequences under study were aligned at the amino acid and nucleic acid levels to determine regions that show maximum similarity (Fig. 1). These "islands" of homology were used to design several test sets of primers that were evaluated for their ability to retrieve all members of closest-homology subgroupings (i.e. subfamilies) with equal efficiency. These test amplifications were performed from a set of cloned templates representing the entire target collection diluted to equivalent molar (target number) ratios. When a primer pair was found capable of amplifying a gene subfamily, the analogous region from the global alignment was compared and amplimers made and tested to retrieve the respective subfamily members.

Clustal alignments of the Kir channel gene family revealed all members to possess a high degree of sequence similarity in core regions [9]. For the purposes of isoform profiling, we selected two conserved motif sequences in the pore and mid C-terminal regions (asterisked in Fig. 1) which we refer to as the G(Y/F)G and the ILE motifs, in accordance with the predicted amino acid sequence encoded by them. These initial alignments included the isoforms hKir1.1, hKir2.1, hKir2.2, hKir2.3, rKir2.4, hKir3.1, rKir3.2, hKir3.3, hKir3.4, hKir4.1, hKir4.2, rKir5.1, hKir6.1, hKir6.2 and rKir7.1 where the h- prefix indicates a human-derived sequence and the r- prefix a rat-derived sequence. If a human-derived sequence was available, we used it in preference to rat for the initial alignments; alternatively, for derivation of profiling data, we isolated the corresponding Kir channel cDNA from a human leukocyte cell source and determined the sequence independently. Sub-family centred aligned sequences of mixed vertebrate species origin thus permit derivation of the highly conserved motifs. Having established the cognate sequences of human Kir isoforms, it was possible to derive a series of human-cognate 'bracketing' primers that encompassed the G(Y/F)G- and ILE-motif segments (Fig. 2).

Verification of sub-family centred primer pairs

Clones of each of the Kir isoforms were generated by amplification from human genomic DNA or mRNA using the specific primer pairs described in Fig. 2, and were used as cognate, positive control templates for downstream degenerate RT-PCR. Sub-family centred degenerate PCR primers were designed to map to the G(Y/F)G and ILE regions which are highly conserved throughout the Kir channel family and are shown in Table 1. The degenerate primer pairs were verified to amplify a 500 bp band from their cognate sequences (Fig. 3). Cross-reactivity of the primers to non-cognate sequence was limited with amplification of the 500 bp band detectable in only four from eighty-four reactions (Fig. 3).

To ensure that amplification was from mRNA as opposed to contaminating genomic DNA, total RNA was treated with DNase I prior to RT-PCR. Treatment with DNase I was found to completely prevent amplification of the 500 bp product from 10 μg of cognate DNA template in a 40 cycle PCR reaction (Fig. 4). Titration of DNA concentration indicated saturation of the PCR reaction after 40 cycles and independence of template concentration (Fig. 4).

Restriction digest of degenerate PCR product generates Kir isoform-specific banding patterns

ScrF I digests were performed upon the positive control degenerate PCR products and it was shown that the subfamily centred PCR-restriction digest protocol was capable of producing a diagnostic Kir isoform-specific duplex banding pattern in agreement with that predicted from the published human sequences (Fig. 5). Potential problems were encountered as low molecular weight restriction fragments i.e. <50 bp were found to be less detectable and spurious bands were apparent. This was probably as a result of the disruption of potential restriction sites due to the T4 DNA polymerase end-labelling scheme and associated 3'-5' exonuclease activity, or the degeneracy of the primer sequences.

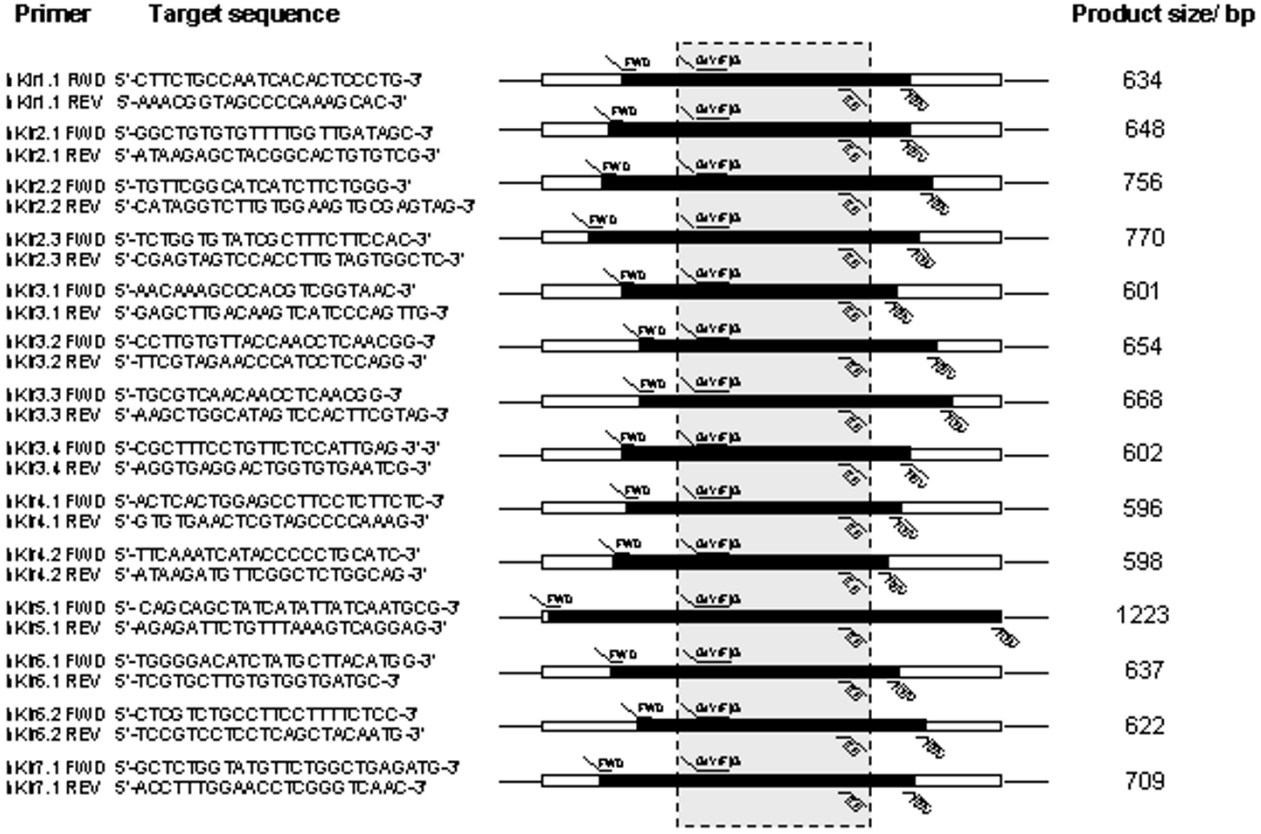

Figure 2
Definition of human Kir isoform specific primers. PCR primers specific for human Kir isoforms were designed to bracket the homologous motifs, G(Y/F)G and ILE. Sequence of forward (FWD) and reverse (REV) primers are described, and the regions of Kir targeted are indicated.

Mast cells, eosinophils and neutrophils have a distinct pattern of Kir isoform expression

Application of the above protocol to mRNA derived from highly purified HLMC (Fig. 6A), eosinophils (Fig. 6B), and neutrophils (Fig. 6C) demonstrated distinct Kir isoform expression, based on the predicted restriction fragment patterns illustrated in Figure 1. All three leukocytes subsets generated fragment sizes consistent with the expression of Kir 2.1 (478 bp), 2.2 (49 bp) and 2.3 (298 bp) mRNA (Fig. 6). Potential evidence of Kir 2.4 expression was also observed in each leukocyte based on the detection of a band of approximately 250 bp consistent with the predicted size for Kir 2.4 (Fig 1). However, absolute determination of Kir 2.4 expression was not possible in the absence of a positive control for this isoform (Fig. 3). Mast cells and neutrophils also had similar expression of Kir 3.0 isoforms, with a banding pattern consistent with Kir 3.1 and 3.2 mRNA expression, as determined by the detection of 85 bp and ~30 bp fragments respectively. The ~250 bp fragment was derived from both Kir 3.1 and 3.2. Neutrophils alone exhibited detectable expression of Kir 4.2 as evidenced by the 350 bp fragment (Fig. 6C). Both HLMC and eosinophils expressed members of the Kir6.0 family, with bands consistent with the presence of Kir 6.1 (274 bp) and 6.2 (325 bp) in mast cells, but only Kir 6.1 in eosinophils.

Improved detection of low molecular weight restriction fragments by modified Klenow end-labelling

Due to previous problems observed in the lack of clear labelling of low molecular weight terminal restriction fragments (Figs. 5), a protocol utilising DNA polymerase I large (Klenow) fragment, which lacks 5' to 3' exonuclease activity whilst retaining 5' to 3' polymerase activity, in conjunction with a probe with a sub-family specific 3' end and 10x G residues at the 5' end was developed. Annealing of the probe with the 500 bp degenerate PCR product and subsequent 5' to 3' DNA polymerase I large (Klenow) frag-

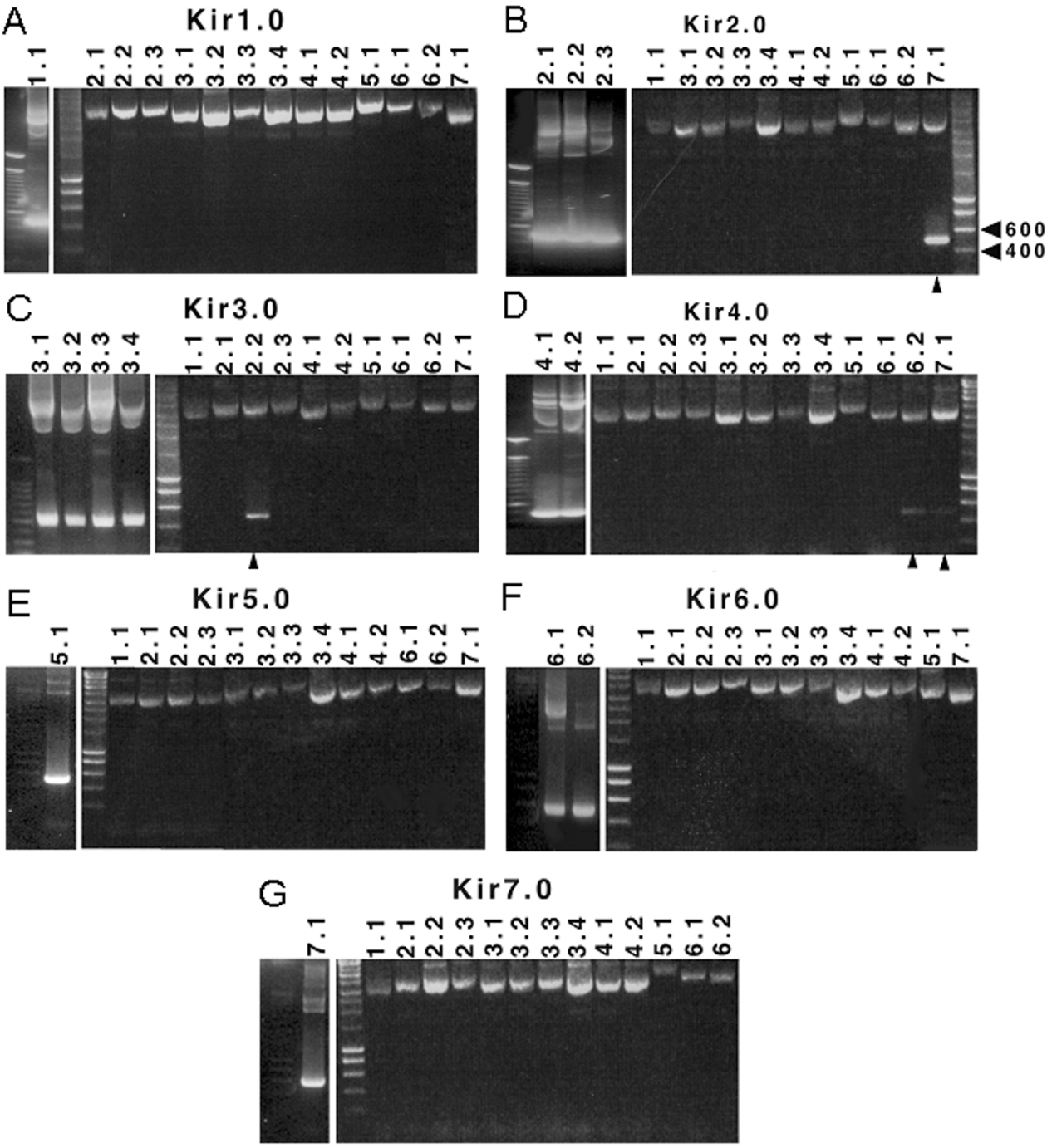

Figure 3
Validation and cross-reactivity of degenerate sub-family centred primers. 40 cycle PCR reactions were set up using degenerate sub-family centred primers against both non-cognate and cognate (positive control, left hand gel (a)-(g) clonal template. Detectable cross-reaction is indicated (*arrowhead*). Gel images were scanned and processed using Adobe Photoshop Version 5.5.

Table 1: Sub-family centred primer design.

hKir	Degenerate primer sequence
1.0	FWD 5'-GCAAGTGACCATT**GGATATGGA**-3' REV 5'-GCACTGTGCC**ATCTAAAAA**CAC-3'
2.0	FWD 5'-GVCAGACVWCCATM**GGCTAYGG**-3' REV 5'-GMACCATRCC**YTCMAGDAT**GAC-3'
3.0	FWD 5'-GVGARRCMACCATY**GGBTAYGG**-3' REV 5'-GCACMATBCC**YTCKAGRAT**RAC-3'
4.0	FWD 5'-GCCARACMACCATT**GGCTATGG**-3' REV 5'-GCACWGTSSC**AYTKAGGAK**SASCAC-3'
5.0	FWD 5'-GCAAACCACCATA**GGATATGGT**-3 REV 5'-GACCAGTATA**GATAAATGT**CAC-3'
6.0	FWD 5'-GYCAAGTKACYATT**GGSTTTGG**-3' REV 5'-GMACCACKCC**TTCCAGRAT**RAC-3'
7.0	FWD 5'-GCAACTCACAATT**GGTTATGGT**-3' REV 5'-GCTGCATTGC**TGAAAGGAA**TAC-3'

Degenerate base key: B, C or G or T; D, A or G or T; K, G or T; M, A or C; R, A or G; S, C or G; V, A or C or G; W, A or T; Y, C or T. Degenerate, 'sub-family centred' PCR primers were designed with the regions of homology, G(F/Y)G and ILE, towards the 3' ends of the forward (FWD) and reverse (REV) primers respectively (*bold*). A non-homologous 5' G residue was added to each primer to facilitate potential fluorescent end-labelling of the PCR product.

ment activity increased ten fold the incorporation of [α^{32}P] dCTP. This enabled clear detection of low molecular weight restriction fragments when applied to the Kir 2.0 sub-family (Fig. 7). Restriction enzyme Hinf I was used in this instance as it generated a more distinct digestion pattern for the Kir2.0 subfamily in isolation than the previously used ScrF1. Using this end-labelling protocol and restriction digestion with Hinf I, Kir 2.1, 2.2, and 2.3 would generate fragments of predicted size of 147, 400, and 55 bp respectively. Therefore the protocol allowed clear discrimination of the Kir 2.0 isoforms (Fig. 7).

Discussion

In the present study we have devised a novel, rapid, and flexible strategy for the determination of gene family isoform expression in any cell type, and have used it to examine the expression of the Kir family of K^+ channels in human leukocytes.

The overriding aim was to limit the number of RT-PCR reactions required to identify all family members while at the same providing a reliable "readout" of genes expressed. Single PCR reactions for each Kir channel would entail 15 individual PCRs, with each potentially different optimal conditions, but by designing degenerate primers for each subfamily, capable of amplifying all appropriate subfamily members, we were able to limit the number of PCR reactions to seven. Furthermore, by generating primers aimed at producing the same size product for all 7 families, we were able to employ a single set of PCR conditions throughout. The final PCR product for a subfamily could contain mRNA from up to 4 channels in the case of the Kir 2.0 and 3.0 families. By using restriction enzyme digestion and subsequent electrophoresis, specific DNA fingerprints were predicted and confirmed for each subfamily, thus increasing the specificity of the final result compared to stand-alone RT-PCR.

DNase I treatment crucially prevented the amplification of intronless genes such as the Kir 2.0 family from contaminating genomic DNA. With the exception of Kir 2.4, we were able to clone template for use as a positive control to demonstrate primer activity, selectivity, and subsequent restriction digest fingerprinting. Of note, we demonstrated the potential for cross-reactivity between a few subfamily members, for example the Kir 4.0 degenerate primers amplify Kir 6.2 and Kir 7.1 (Fig. 3), which need to be taken into account if bands of unpredicted size are observed within a particular subfamily. Such cross-reactivity between closely related isoforms is a potential problem of the profiling strategy that can be at least partially overcome by extension of the degenerate oligonucleotide primers. Potential problems with detection of low molecular weight restriction fragments i.e. <50 bp were overcome using the modified DNA polymerase I large (Klenow) fragment end-labelling, but ideally the use of radiolabelled or fluorescently labelled degenerate PCR primers would obviate downstream labelling reactions and help solve such problems.

We have demonstrated quite clearly that this method for detecting multigene family expression is applicable to the study of Kir gene expression in human leukocytes. The cells studied were highly purified, but there is always the chance that a PCR product has been derived from a contaminating cell, so the results from the initial PCR should be considered as a guide to be confirmed by specific identification of protein. Nevertheless the usual contaminating cells in an eosinophil preparation are neutrophils, and vice versa, and since we have identified expression of different families in these 2 cell types, contamination in this case is unlikely. Interestingly, we observed potential expression of all Kir 2.0 family members in all three cell types studied, Kir 3.1 and 3.2 in both HLMC and neutrophils, and Kir 4.2 in neutrophils alone. In addition,

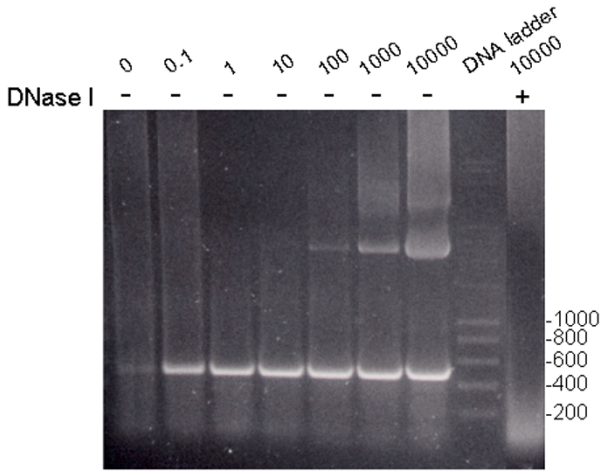

Figure 4
DNase I treatment of template prevents amplification of 500 bp product. Series of PCR reactions were performed on varying concentrations of clonal template DNA. Mass of template DNA (ng) and DNase I treatment (+/-) is indicated above the lanes. DNA ladder was run as molecular weight standard with sizes indicated (bp). Treatment with DNase I as described in *Materials and Methods* prevented amplification of 500 bp product from 10 μg of DNA. Gel image was scanned and processed using Adobe Photoshop Version 5.5.

HLMC expressed Kir 6.1 and 6.2, while eosinophils exhibited sole expression of Kir 6.1. Using patch clamp recording we have been unable to identify any Kir-like currents in HLMC (manuscript submitted), but as mast cells require activation by stem cell factor for survival and Kir 2.0 channels may be closed by tyrosine phosphorylation [11], it is possible that the channels are closed under basal conditions. Our results therefore suggest that a more detailed investigation of HLMC using inhibitors of tyrosine kinases may be worthwhile. The inwardly rectifying K^+ current in eosinophils has electrophysiological properties consistent with the presence of Kir 2.1, and the cells express mRNA for Kir 2.1 [6]. Other Kir isoforms were not looked for in this study, but our results suggest that other Kir 2.0 family members may also be present. Recently it has been suggested that eosinophils also express ATP-sensitive (Kir 6.0 family) K^+ channels [7], so our observation of Kir 6.1 mRNA expression in eosinophils is compatible with this. In neutrophils an uncharacterised inwardly-rectifying K^+ current is present at rest [8], and our results would suggest again that Kir 2.0, Kir 3.0 or Kir 4.0 family members could contribute to this. Expression of mRNA does not necessarily mean that there is translation into protein or that translated protein is functional, but nevertheless provides a powerful tool for initial screening. Therefore, patch clamp recording with appropriate tools to modulate these channels, study of protein expression when suitable antibodies to the human Kir channels become available, and an antisense deoxynucleotide approach to "knockout" channel function as applied to Kir 2.1 in rat ventricle [9], will be required to determine the specific roles of the Kir channels in these leukocytes.

A particular strength of the method is that not only can we detect all currently known subfamily isoforms, but also there is the potential to amplify novel hitherto-unidentified family members. Interestingly, in the leukocytes tested there was an identical banding pattern for the Kir 2.0 family, with evidence for expression of Kir 2.1–2.4. Also, there was a consistent band of 375 bp which was unaccountable, and which was not consistent with a cross-reaction from another subfamily, raising the possibility that it could be derived from a novel Kir 2.0 family member (Fig. 3). Obviously the generation of this and other novel fragments being due to partial restriction digestion cannot be ruled out. However, we have previously shown that target amplification was independent of template copy number (Fig. 4). Therefore one would expect fragments derived from novel isoforms to be of similar abundance as known isoforms, whereas incomplete digestion would result in bands of markedly reduced abundance comparative to complete digestion products.

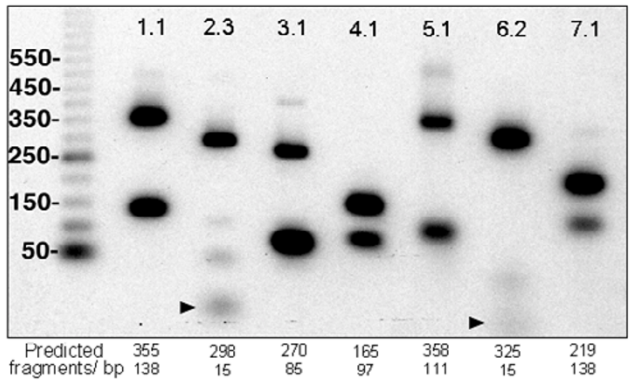

Figure 5
Validation of Kir isoform typing with reporter enzyme ScrF I. Degenerate PCR with sub-family centred primers was performed on Kir subclone templates and products were T4 DNA polymerase end-labelled according to *Materials and Methods*. Diagnostic restriction digests were performed using ScrF I and an isoform specific duplex banding pattern generated consistent with the predicted banding patterns described in Figure 1. Faint low MW fragments (<50 bp) are indicated with an arrowhead. One Kir isoform from each sub-family is shown. The predicted fragment sizes based on sequence analysis for each isoform are indicated (bp). Gel image was scanned and processed using Adobe Photoshop Version 5.5.

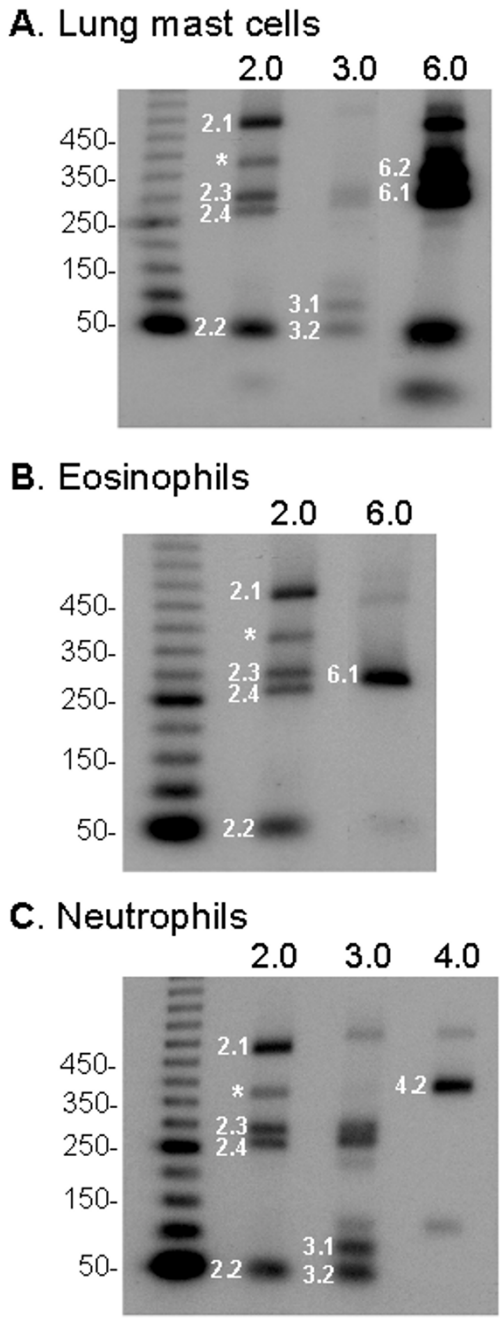

Figure 6
Isotyping of Kir expression in eosinophils, neutrophils and lung mast cells. Lung mast cells (A), eosinophils (B), and neutrophils (C) were purified and Kir isoform profiling performed according to the strategy described in the text. 50 bp DNA ladder was run as molecular weight standard with sizes indicated (bp). Potential correlation with Kir isoforms is indicated to the left of the appropriate fragment. *Fragment of unknown origin. Results are representative of two independent experiments. Gel images were scanned and processed using Adobe Photoshop Version 5.5.

Conclusion

We have developed a multigene isoform profiling strategy and described its application when applied to the study of inwardly rectifying K^+ channel gene expression in human leukocytes. A similar approach will simplify the investigation of tissue and cell-specific expression of other ion channel and multigene families in the future.

Methods

Reagents and materials

Kit for DNA isolation from peripheral blood cells, T vector, RQ1 RNase-free DNase I, T4 DNA polymerase I, and DNA polymerase I large (Klenow) fragment were from Promega (Southampton, UK). AdvanTaq™ polymerase was purchased from Clontech (Hampshire, UK). Oligonucleotide primers were generated by the Protein and Nucleic Acid Chemistry Laboratory (Leicester University, UK). All other reagents were from Sigma (Poole, UK) unless otherwise stated.

Isolation of peripheral blood eosinophils and neutrophils

Heparinised peripheral venous blood was taken from healthy volunteers with peripheral blood eosinophilia of less than 0.5×10^6 ml^{-1}. Granulocytes were purified by Phycoll density gradient centrifugation, with eosinophils further purified by negative immunomagnetic selection as described previously [12]. Briefly, removal of erythrocytes by dextran sedimentation was followed by centrifugation of the leukocyte rich supernatant (100 *g* for 15 min at room temperature). The cell pellet was resuspended in Hanks' balanced salt solution without Ca^{2+} and Mg^{2+}, supplemented with 1% BSA and 20 mM EDTA. The cell suspension was centrifuged on Histopaque 1083 and the mononuclear cell layer carefully removed prior to lysis of erythrocytes contaminating the granulocyte pellet by hypotonic shock using sterile, ice-cold water. Eosinophils were separated from predominating neutrophils by negative immunomagnetic selection using anti-CD16-coated magnetic beads (Miltenyi Biotec Inc., Auburn, CA, USA). Post-isolation purity and viability was routinely >98% as assessed by morphology after Kimura stain and trypan blue exclusion respectively.

Isolation of human lung mast cells

HLMC were dispersed from macroscopically normal lung obtained within 1 h of resection for lung cancer as described previously [13]. Mast cells were purified using immunomagnetic affinity selection with anti-mouse IgG_1 magnetic beads (Dynal, Wirral, UK) coated with the mouse anti-*c-kit* mAb YB5.B8 (Cambridge Bioscience, Cambridge, UK). Final mast cell purity was >99% and viability >97%.

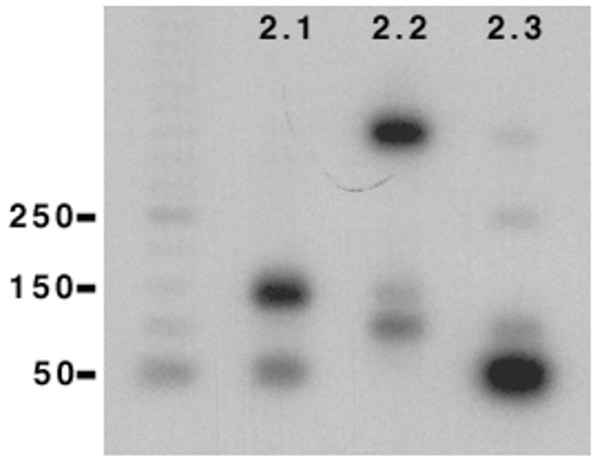

Figure 7
Increasing efficiency of detection of low MW restriction fragments. Using Klenow DNA polymerase and a probe with a sub-family specific 3' end and 10x G residues at the 5' end, the efficiency of the labelling reaction could be increased ten fold. Degenerate sub-family centred PCR was performed using the Kir 2.0 subclones as template, labelled as described in *Materials and Methods*, and digested with Hinf I. Low molecular weight fragment (<50 bp) end-labelling was significantly improved. Generated end-labelled fragments from Kir 2.1, 2.2, and 2.3 were in agreement with predicted sizes of 147, 400, and 55 bps respectively. Gel images were scanned and processed using Adobe Photoshop Version 5.5.

DNA isolation

Human genomic DNA from peripheral blood cells was isolated using a kit according to manufacturers instructions (Promega, Southampton, UK), precipitated with isopropanol and pelleted at 15000 *g* for 1 min at room temperature. Plasmid DNA was extracted using a kit from Gibco BRL (Paisley, UK), precipitated with isopropanol and pelleted at 15000 *g* for 1 h at 4°C. DNA was washed briefly in 70% ethanol and resuspended in TE (10 mM Tris, pH 7.6, 1 mM EDTA)

DNA constructs

Kir channel cDNAs were cloned from human genomic DNA into T vector using cognate specific primers, and transformed into competent *Escherichia coli* cells (TOP10, Invitrogen, CA). Human Kir 3.0 clones were a kind gift from Dr. Van Tol (University of Toronto, Ontario, Canada).

mRNA analysis

RNA was extracted using TRI Reagent (Sigma, Poole, UK) and stored in 70% ethanol at -70°C. PolyA+ RNA was isolated with Dynabeads oligo $(dT)_{25}$ according to the manufacturers protocol (Dynabeads, Dynal, Oslo, Norway) and eluted into DEPC-treated water. mRNA was treated with RNase-free DNase I for 30 min at 37°C, followed by heat inactivation at 65°C for 10 min, and reverse transcription with *Superscript* RT (Gibco BRL. Paisley, UK) for 1 h at 42°C, in the presence of 100 mM DTT, 10 mM dNTP and RNase inhibitor. cDNA product was amplified by polymerase chain reaction (PCR), in a 50 μl PCR reaction containing 5 μl cDNA, 20 pmol of each sub-family centred primer, 0.2 mM dNTP, 2.5 units AdvanTaq™ DNA polymerase. Amplification consisted of 40 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 1 min, and a final extension step of 72°C for 7 min. Amplification products were electrophoresed on 1.2% agarose gels and visualised by ethidium bromide staining under UV and photographed.

T4 DNA Polymerase end-labelling of degenerate PCR products

The products from the degenerate, sub-family centred PCR were excised and purified from agarose gels using a kit (Hybaid Ltd, Middlesex, UK) according to manufacturers protocol, and eluted into 20 μl elution solution. For T4 DNA polymerase end-labelling a fraction of the purified PCR product was combined with reaction buffer to a final concentration of 1X, 100 μM dATP, dTTP and dGTP, 5 μCi [α-^{32}P] dCTP (3000 Ci/mmol), 1 unit of T4 DNA polymerase I and water to a final volume of 20 μl. Reaction was incubated at room temperature for 10 min, and stopped by heat inactivation at 75°C for 10 min. Labelled DNA was purified from unincorporated [α-^{32}P] dCTP using Hybaid DNA purification kit, and eluted in 20 μl H_2O.

DNA polymerase I large (Klenow) fragment end-labelling of degenerate PCR products

The products from the degenerate, sub-family centred PCR were excised and purified from agarose gels using Hybaid DNA purification kit and eluted into 20 μl elution solution. 5 μl of DNA was annealed to 20 pmol of modified degenerate PCR primer at 94°C for 1 min and cooled to room temperature for 5 min. The degenerate PCR primer was modified by the addition of ten non-homologous 5' G residues, thereby facilitating [α-^{32}P] dCTP labelling of the 5' protruding end by the 5' to 3' polymerase activity of DNA polymerase I large (Klenow) fragment. The annealed template was labelled using 5 units of DNA polymerase I large (Klenow) fragment, 1X reaction buffer, 5 μCi [α-^{32}P] dCTP (3000 Ci/mmol) and water to a final volume of 50 μl, and incubated at room temperature for 1.5 h, prior to heat inactivation at 75°C for 20 min. Using this protocol only one end of the degenerate PCR product was labelled. To remove unincorporated [α-^{32}P] dCTP, the reaction mix was carefully applied to a pre-spun G-50 microspin column (Amersham-Pharmacia Biotech, Little Chalfont, UK) and centrifuged at 735 *g* for 2 min.

Diagnostic digestion of end-labelled DNA

The purified T4 DNA polymerase end-labelled DNA was restriction digested with 5 units of ScrF I in a final volume of 30 μl, for 4 h at 37°C. The Klenow DNA polymerase labelled DNA was restriction digested with 5 units of Hinf I in a final volume of 30 μl, for 4 h at 37°C. 2 μl of loading dye was added to the restriction reaction and electrophoresed through 2.0% agarose for 3.5 h in Tris-acetate buffer (40 mM Tris, 1 mM EDTA, 0.02 M acetic acid) without ethidium bromide. The gel was then dried down with heat under a vacuum onto Whatman 3 MM and exposed to photographic film overnight at -80°C.

Authors' contributions

GD carried out the molecular studies, participated in the coordination of the study, and in the drafting of the manuscript. ECC conceived of the study, and participated in its design and coordination. PB participated in the design and coordination of the study, generation of the leukocyte samples, and drafted the manuscript.

All authors read and approved the final manuscript.

Abbreviations

HLMC, human lung mast cells; Kir, inwardly-rectifying family of K^+ channels; RT-PCR, reverse transcriptase-polymerase chain reaction.

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